A Prospective, Randomized Study to Compare the Effect of Combination Therapy With Campath-1H on Peripheral Blood Mononuclear Cells in Kidney Transplant Recipients

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UNRESTRICTED

A PROSPECTIVE, RANDOMIZED STUDY TO COMPARE THE EFFECT OF COMBINATION THERAPY WITH CAMPATH-1H ON PERIPHERAL BLOOD MONONUCLEAR CELLS IN KIDNEY TRANSPLANT RECIPIENTS

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ABSTRACT

Induction therapy with Campath-1H, a humanized anti-CD52 monoclonal antibody depleting T and B lymphocytes, has been used in organ transplantation with the final goal of resetting the immune system in order to promote a tolerance-permissive environment and, at the same time, to reduce the need for chronic maintenance immunosuppression. To explore whether this0 may result from the capability of Campath-1H, in association with different maintenance regimens, to promote regulatory T cells (Treg) expansion and to assess whether this may translate into better graft outcomes in the long-term, 21 renal transplant patients receiving Campath-1H induction were randomized to low-dose SRL (n=11) or low-dose CsA (n=10), both in addition to low-dose mycophenolate mofetil (MMF) as maintenance immunosuppressive therapy and monitored for over 30 month follow-up.

SRL-treated patients showed an important expansion of circulating $CD4^{+}CD25^{high}FOXP3^{+}$ Treg that, at one and two years after transplant, were significantly more abundant than in the CsA group. T cells isolated from peripheral blood long-term post-transplant were hyporesponsive to donor alloantigens in both treatment arms. In SRL-, but not CsA-treated patients, hyporesponsiveness was reversed by Treg depletion. T cells from CsA-treated patients were anergic to donor antigens.

Despite higher Treg counts, SRL-treated patients had a faster GFR and RPF decline, more clinical proteinuria, significantly higher tubular C4d staining score and a trend to higher chronic allograft damage index score, compared to CsA-treated patients. There was no significant correlation between Treg counts and any considered outcome variable in the study group as a whole and within each treatment group.

These data suggest that, after Campath-1H induction, maintenance therapy with low-dose SRL and MMF promotes Treg expansion, but this is not paralleled by long-term improved graft outcomes. Conversely, maintenance immunosuppression with low-dose CsA and MMF is associated with better graft function and structure than low-dose SRL plus MMF, possibly through the induction of T cell anergy toward donor antigens.

AIMS

Primary

1. To compare the effect of Campath-1H, low-dose sirolimus versus Campath-1H, low-dose CsA, both in addition to low-dose MMF on phenotypic and functional profiles of peripheral blood mononuclear cells (PBMCs) in kidney transplant recipients.

2. To assess whether the increased counts of circulating $CD4^{+}CD25^{high}$ Treg may translate into better graft outcomes in the long-term.

Secondary

To compare in the two groups of kidney transplant recipients:

- Incidence of acute allograft rejection
- Time course of graft function (as serum creatinine concentration)
- Time course of glomerular filtration rate and renal plasma flow (measured by iohexol clearance and p-amminohippurate, respectively)
- Systolic and diastolic blood pressure
- Lipid profile (cholesterol, triglycerides, HDL)
- 24 h urinary protein excretion rate
- Patient and graft survival
- Incidence of major adverse events.

INTRODUCTION

1. Kidney transplantation: past, present, and a possible future

An historical perspective

December 23, 1954, represents a milestone date for organ transplantation. That day, a surgical team under the direction of Joseph Murray, removed a kidney from a healthy donor and transplanted it into his identical twin, who had chronic renal failure secondary to glomerulonephritis [1, 2]. The organ functioned immediately, and the recipient survived for nine years, when his allograft failed from recurrent glomerulonephritis. The donor has survived for 50 years [1, 2].

As more transplantations were performed between identical twins [3], approaches to suppressing the recipient's immune system were pursued so that transplantation might be extended beyond procedures involving identical twins. The knowledge in immunology however was still rudimentary. The first attempt to suppressing the rejection process, taken in the early 1950s, involved the use of sublethal total-body irradiation combined with cortisone. These attempts failed in most of the cases, with the exception of some transplantations between nonidentical twins - first at Peter Bent Brigham and a few weeks later in Paris [1, 4] - which provided the impetus to search for more effective ways to prevent rejection.

A major contribution in the control of acute rejection was provided by Robert Schwartz and William Dameshek, hematologists at Tufts University School of Medicine, who reported that 6-mercaptopurine (6-MP), which was already in clinical use for the treatment of acute lymphocytic leukemia, suppressed the immune response in rabbits [5, 6]. The Wellcome Research Laboratory then synthesized several variants of 6-MP for screening by Joseph Murray and Roy Calne in dog kidney transplantations. Only one

candidate drug, azathioprine, resulted in long-term survival and in only a small number of animals. These observations prompted a rather anxious start to the first clinical trial, in 1962, of chemical immunosuppression involving azathioprine [7]. In patients in whom azathioprine was combined with a corticosteroid, one-year rates of allograft survival were in the range of 40 to 50 percent, an enormous improvement over previous results. These clinical breakthroughs were ultimately recognized by awarding of Nobel Prizes to Joseph Murray (and others), for the first clinical transplantation and the first use of immunosuppression, and to George Hitchings and Gertrude Elion of the Wellcome Laboratory, for the development of drugs, including azathioprine, that affect nucleotide pathways [1].

The rate of successful transplantation of kidneys from cadaveric donors and familial HLA-matched living donors slowly increased during the 1960s and early 1970s, following the introduction of azathioprine with corticosteroids. Although the initial effect was beneficial, prolonged use of corticosteroids resulted in a high mortality rate due to excessive immunosuppression. Overall mortality rates also fell as programs for long-term dialysis improved, which made it possible to discontinue immunosuppression and sustain life when grafts failed [1]. In the late 1970s, cyclosporine was introduced, which increased the rate of one-year graft survival from 70 percent to more than 80 percent [8]. During last decades, results of kidney transplantation have been improved to the point that this procedure is now considered the ideal treatment for patients with end stage renal disease.

Three-Signal Model of Alloimmune Responses

In 1925, Emile Holman, a surgeon at Peter Bent Brigham Hospital who performed skin grafts in children with extensive burns, reported that repeated grafts from maternal donors were rejected more rapidly than the initial grafts, which indicated donor-specific sensitization to the "proteins" of the donors [1]. These proteins were in the following decades identified as HLA antigens and the immune reaction against these alloantigens was thereafter named as rejection.

Alloimmune responses involve both naive and memory lymphocytes [9], including lymphocytes previously stimulated by viral antigens cross-reacting with HLA antigens[10]. In the graft and the surrounding tissues, dendritic cells of donor and host origin become activated and move to T-cell areas of secondary lymphoid organs. There, antigen-bearing dendritic cells engage alloantigen-reactive naive T cells and central memory T cells that recirculate between lymphoid compartments but cannot enter peripheral tissues [11]. Naive T cells are optimally triggered by dendritic cells in secondary lymphoid organs [12, 13], but antigen-experienced cells may be also activated by other cell types, such as graft endothelium [14].

An antigen on the surface of dendritic cells that triggers T cells with cognate T cell receptors constitutes "signal 1," transduced through the CD3 complex. Dendritic cells provide costimulation, or "signal 2," delivered when CD80 and CD86 on the surface of dendritic cells engage CD28 on T cells. Signals 1 and 2 activate three signal transduction pathways: the calcium–calcineurin pathway, the RAS–mitogen-activated protein (MAP) kinase pathway, and the nuclear factor-κB pathway [15]. These pathways activate transcription factors that trigger the expression of many new molecules, including interleukin-2, CD154, and CD25. Interleukin-2 and other cytokines (e.g., interleukin-15) activate the mammalian target of rapamycin (mTOR) pathway to

provide "signal 3," the trigger for cell proliferation. Lymphocyte proliferation also requires nucleotide synthesis. Proliferation and differentiation lead to a large number of effector T cells [16]. B cells are activated when antigens engage their antigen receptors, usually in lymphoid follicles or in extrafollicular sites, such as red pulp of spleen[17], or possibly in the transplant [18], producing alloantibody against donor HLA antigens. Thus, within days the immune response generates the agents of allograft rejection, effector T cells and alloantibodies.

HLA antigen presentation: direct and indirect pathways

Alloreactive T cells recognise alloantigens via two distinct pathways: direct and indirect. Direct recognition occurs when recipient T cells recognise intact donor MHC molecules complexed with peptide on donor stimulator cells. In contrast, indirect recognition occurs when the recipient APC process the donor-MHC molecules prior to presentation to recipient T cells in a self-restricted manner [19].

The first clear evidence that T cells with exclusively direct allospecificity can effect transplant rejection was provided by a relatively recent study. Reconstitution of SCID or $Rag1^{-/-}$ mice with syngeneic $CD4^+$ T cells led to rejection of MHC class II-expressing cardiac allografts but not MHC class II-deficient grafts [20]. Furthermore, $Rag1^{-/-}$ mice that were also MHC class II-deficient rejected allogeneic cardiac transplants when reconstituted with $CD4^+$ T cells. Since these mice have no $CD8^+$ cells and lack the capacity for MHC class II-restricted indirect allorecognition, these results indicate that direct pathway $CD4^+$ T cells were both necessary and sufficient to mediate allograft rejection [20].

Anti-donor alloreactive T cells derived from the naïve fraction of the recipients' T cell repertoire must be primed in lymphoid tissue. Therefore, the priming of naïve direct pathway alloreactive T cells is likely to only occur predominantly during the first few weeks after transplantation, while donor-derived dendritic cells are available. Once those dendritic cells have died, the naïve T cell repertoire of the recipient is likely to be less important as far as direct anti-donor responses are concerned [19]. Intriguingly, Lechler and Batchelor showed that injection of donor-derived dendritic cells is able to restore immunogenicity of rat renal grafts depleted of incompatible passenger cells [21, 22].

The hypothesis that also indirect pathway may play a role in transplant rejection was first proposed by De la Rosa and Talmage in early 1980s [23]. Ten years later, Auchincloss et al. clearly confirmed the importance of this pathway in allogeneic response by using MHC deficient mice [24]. Their most compelling evidence that the indirect pathway is sufficient to mediate transplant rejection was the observation that MHC class I knock out recipient mice could reject skin grafts from MHC class II knock out donor mice [24]. The recipient mice lacked $CD8^+$ cytotoxic T cells capable of recognising donor MHC class I molecules directly, and the $CD4^+$ T cells in the recipient animals could only be stimulated by recognising donor MHC class I molecules indirectly, presented in the context of recipient MHC class II molecules. These findings were supported using a murine skin allograft model, which demonstrated that the indirect pathway alone was sufficient to elicit allograft destruction in the absence of direct allorecognition [25]. Primed cells from mice previously immunised with allogeneic spleen cells or skin cells were shown to proliferate in response to peptides derived from donor-MHC in the context of self-MHC.

Notably, many studies have suggested that the direct and indirect pathways of allorecognition engage in cross-talk, for example $CD4^+$ T cells with indirect antidonor specificity can amplify direct pathway $CD8^+$ T cell responses and direct pathway $CD8^+$ T cells can also be regulated by tolerant indirect pathway $CD4^+$ T cells [26]. Moreover, Lechler et al. recently described another mechanism by which alloreactive T cells are activated. Recipient DCs can acquire donor MHC through cell-to-cell contact and this acquired MHC can stimulate a T cell response, which has been called the semidirect pathway [27].

Effectors and Lesions of Rejection

Effector T cells that emerge from lymphoid organs infiltrate the graft and orchestrate an inflammatory response, which recruit activated macrophages, B cells, and plasma cells that eventually induce parenchymal injury and deterioration of graft function [16]. The diagnostic lesions of T cell–mediated rejection reflect mononuclear cells invading the kidney tubules (tubulitis) and the intima of small arteries (arteritis). [28]. Injury is not simply lysis of target cells, since typical lesions develop in mice lacking cytotoxic T-cell lytic molecules, but may involve transdifferentiation of epithelial cells into mesenchymal cells[15] and cell senescence [28].

A humoral reactivity against donor antigens may also occur, eventually resulting in antibody-mediated acute rejection. This is diagnosed by clinical, immunologic, and histologic criteria, including a demonstration of complement factor C4d in peritubular capillaries. C4d is a fragment of the classical complement pathway component C4, which is activated by antigen-antibody complexes [29]. C4 is activated and proteolytically cleaved into C4a and C4b, exposing a reactive and short-lived thiolester

group in C4b that binds to nearby molecules covalently. C4b is subsequently inactivated by cleavage into C4c and C4d, the latter fragment containing the covalent bond to the tissue that thereby can remain at the site of complement activation [29].

Humoral response may also be mild and not induce an acute injury. The presence of anti-HLA antibodies, however, has been associated with the development of chronic rejection [30]. Chronic rejection, clinically defined as progressive loss of renal function with hypertension and low-grade proteinuria, is the leading cause of late allograft dysfunction and accounts for renal failure in 50 to 80% of recipients who return to dialysis after transplantation [31]. The morphologic diagnosis can be difficult. The two most definitive features of chronic renal allograft rejection are the arterial intimal thickening with mononuclear cell infiltration and the duplication of the glomerular basement membrane (GBM). However, the arterial lesion preferentially affects the larger vessels, which are not always sampled in needle biopsies. The glomerulopathy can be mild or focal and by itself is not pathognomic of chronic rejection because it is also seen in thrombotic microangiopathy and certain chronic immune complex diseases. Interstitial fibrosis and tubular atrophy are similarly nonspecific findings that are compatible with a variety of causes, including post ischemic injury, hypertension, and chronic cyclosporine toxicity [31]. The inability to distinguish these conditions by histologic characteristics is reflected in the literature by the nonspecific term chronic allograft nephropathy, which has been used to encompass the end result of chronic injury resulting from immunologic reaction to donor alloantigens as well as from nonimmunologic mechanisms [32, 33]. More recently, an even more generic definition has been chosen for this condition by the Banff '05 Meeting: Interstitial fibrosis and tubular atrophy, no evidence of any specific etiology [34].

2. The hurdle of long-term immunosuppression and the importance of tolerance induction in organ transplantation

Newly developed immunosuppressive drugs have led to combination therapies that have significantly lowered the rates of acute rejection [16]. Moreover, induction protocols with various antilymphocyte antibodies also reduced the rate and intensity of acute rejection [35]. All immunosuppressive drugs have specific side effects and additively contribute to an overall state of immunosuppression, which leads to an increased risk of opportunistic infections and malignancies [16]. Calcineurin and mTOR inhibitors are also frequently associated with hypertension and impaired glucose and lipid metabolism, which eventually may contribute to increase the cardiovascular disease, which is the most common cause of premature death in transplant recipients [36]. In addition, the intrinsic nephrotoxic effect of immunosuppressive drugs such as calcineurin inhibitors promotes a progressive renal function deterioration [37]. Of note, also drugs previously considered devoid of any nephrotoxicity such as mammalian target of rapamycin (mTOR) inhibitors have been more recently demonstrated to negatively affect both glomerular and tubular cells [38].

This, together with the extension of donor and recipient criteria for transplantation [39, 40], might explain why long-term graft outcomes did not change appreciably during last decade, in spite of a remarkable reduction of acute rejection rates in the short period. Indeed, results from the United Network for Organ Sharing (UNOS) database analyses showed that, at 1 year after transplantation, graft survival has reached levels higher than 90% [41]. However, despite the significant decrease in overall acute rejection rates, the improvements in long-term graft survival in the last decade have been still limited [42].

All these issues provide a strong rationale for developing strategies that promote transplantation tolerance.

Defining tolerance

There are many definitions of transplantation tolerance but, in general, it is thought as a condition of stable allograft function without immunosuppression. 'True tolerance' is defined as the absence of any detectable detrimental immune response in an immunocompetent host [43, 44]. The lack of an injurious pathogenic response to the alloantigen is specific, and the recipient is capable of responding to potentially pathogenic microorganisms and malignancies. Importantly, tolerance induction should not only protect the graft from acute rejection, but also from chronic low-grade immune response [44]. Nevertheless, the literature is revealing more and more cases of 'tolerated' grafts actually displaying a histology of chronic rejection. Thus, the term 'operational tolerance', based more on long term stable graft function and absence of exogenous immunosuppression, has been adopted: this less stringent term is often more applicable, particularly to the clinical setting [44].

Spontaneous tolerance in humans

Clinical cases have been reported of kidney transplant recipients whose graft function has been maintained indefinitely after the cessation of immunosuppression, demonstrating that operational tolerance can be achieved [43]. However, these spontaneous phenomena have typically occurred by way of drug nonadherence or withdrawal mandated by complications, and these conditions more commonly lead to

rejection. Thus, transplantation tolerance in humans is a stochastic event under current treatment regimens, but is, in general, possible [43].

Acquired immune tolerance

Seminal studies by Billingham, Brent and Medawar, more than 50 years ago demonstrated that skin grafts from major histocompatibility complex disparate donor mice would be accepted indefinitely when recipient mice had been exposed to donor alloantigen in the neonatal period [45]. These findings provided the proof of the concept that induction of tolerance toward alloantigens is feasible and prompted research of tolerogenic strategies also in the adult animals and in the clinical setting.

Different strategies have been demonstrated effective for tolerance induction in rodent models of transplantation, as well as in pigs and in non-human primates [46-48]. However, tolerance can be more readily achieved in small, inbred animals compared with large, outbred animals, and even robust animal models seem less rigorous than adult humans.

The following paragraphs discuss those mechanisms which, on the basis of experimental studies and some preliminary clinical evidence, may contribute to the induction of transplant tolerance. Importantly, these mechanisms are not mutually exclusive and tolerance may be the result of several of these mechanisms operating simultaneously or sequentially.

Central tolerance

An important characteristic of alloimmune responses is the high frequency of T cells able to recognize and respond to alloantigens. This is at the basis of the common belief

that it is necessary to achieve large-scale deletion of alloreactive T cells in order to create transplantation tolerance [47].

Central tolerance refers to the use of strategies that promote deletion of newly developing T cell with potential anti-donor reactivity within the thymus following encounter with donor derived cells [47].

- Thymic manipulation

The thymus plays an essential role in the maintenance of self-tolerance. Indeed, in spite of its size reduction with age, evidence exists that it remains functional throughout the whole adult life [49]. Intrathymic deletion of self-reactive lymphocytes from the immune repertoire (clonal selection) represents the central mechanism for self-tolerance achievement. This mechanism can be exploited in transplantation by the delivery of donor antigens to the thymus of adult recipients. This may lead to the central elimination of detrimental alloreactive T cell clones, resulting in specific tolerance to donor tissues. Because deletion physically eliminates cells with a certain antigen specificity, it should lead to a robust form of tolerance which, once established, would not be easily perturbed [47]. Hence, deletion would be a desirable tolerance mechanism in the clinical setting. This could either be performed by direct intrathymic injection of donor-derived allopeptides or by peripheral infusion of donor haematopoietic cells that may migrate into the thymus [50].

Many studies have confirmed that intrathymic injection of donor antigen or allopeptides along with peripheral leukocyte depletion may promote operational donor-specific tolerance in rodent models [51, 52]; however, the feasibility of this approach in larger species is still questionable. Furthermore, after the intrathymic delivery of allopeptides,

donor antigen persists in the thymus for only a defined period. Therefore, intrathymic delivery of donor antigen, in contrast to establishment of a stable mixed chimera, provides a transient presence of donor derived antigen and stimulation of tolerant mechanisms, rather than generating persistent deletion of thymocytes. Therefore, additional strategies are needed to control alloreactive T cells, after the intrathymic delivery of alloantigen, to transplant a solid organ graft in the long-term. In animals, the thymus itself has been transplanted in different ways to induce tolerance: as nonvascularized allogeneic thymic tissue, in composite organs ("thymokidney"), and as vascularized thymic lobe transplants [53]. These thymus-dependent strategies might overcome the problem of limited survival of donor cells inside the recipient thymus.

- *Chimerism*

Peripheral infusion of bone marrow (BM) and adult lymphocytes after a conditioning regimen has been attempted to induce a more stable and clinically feasible state of immune tolerance in organ transplantation [54]. Indeed, once infused, donor cells were expected to migrate into the host thymus and mediate selection of alloreactive T cell clones. The conditioning regimen aimed at deleting pre-existing cross-reactive T cells that would reject the donor BM and grafted organ, thus creating 'space' for the engraftment of infused BM cells. However, the potential toxicities for the recipient of initially proposed conditioning protocols made their clinical applicability difficult [55]. Subsequently, the infusion of high-dose donor BM with costimulatory blockade but without prior massive lymphodepletion in the host succeeded in inducing persistent chimerism in mice [56-58]. The inoculus of a larger amount of BM cells allowed overcoming the need of space for donor cells engraftment without the need of

myeloablation, while costimulatory blockade harnessed the peripheral T cell alloresponse.

These experimental evidences formed the basis for the recent successful approaches to induce tolerance in kidney transplant recipients through combined infusion of donor BM [59, 60].

Initial clinical success was reported by Strober using a conditioning regimen based on total lymphoid irradiation (TLI) [61] and has more recently been achieved in patients who require marrow replacement for multiple myeloma [60]. Pilot trials using haplodisparate donor–recipient pairs without underlying malignancy are now ongoing with cautiously optimistic preliminary results [43]. Thus, although practically complex, the induction of mixed chimerism seems to be a promising approach to tolerance.

Recently, two reports have been published on the successful induction of tolerance in patients receiving combined kidney and hematopoietic stem-cell transplantation. The recipient of a kidney from an HLA-matched brother received cyclosporine starting at the time of renal transplantation [59]. During the next 2 weeks, he underwent total lymphoid irradiation, and a course of antithymocyte globulin and prednisone and, therafter, he received an infusion of donor hematopoietic stem cells. Within 1 month after transplantation and consistently thereafter, the proportions of donor and recipient cells in the recipient's blood were about equal. Immunosuppressive therapy was discontinued 6 months after transplantation, with maintenance of good renal function 34 months after transplantation [59]. In another report, Kawai et al. performed simultaneous kidney and stem-cell–enriched leukocyte transplantations from five HLA single-haplotype mismatched living related donors into recipients who had received a conditioning regimen with multiple agents [60]. One patient rejected the kidney. The

other four patients had undefined spontaneously reversible or corticosteroid-responsive "capillary leak" phenomena, which presumably were rejection episodes; nevertheless, immunosuppressive therapy was discontinued in the four recipients 9 to 14 months after transplantation, without deterioration in the function of the grafts during 2.0 to 5.3 years of follow-up. There was no evidence of leukocyte chimerism in any patient for more than 21 days. Since only blood samples were studied, assessment of the presence of small numbers of donor leukocytes (microchimerism) outside the blood circulation was not possible.

These represent important results providing the proof of the concept that tolerance is a feasible goal also in the clinical setting. However, the complexity of the procedure and the important induction immunosuppression still represent major concerns about this strategy.

Peripheral tolerance

Not all self-antigens are expressed in the thymus, thus other mechanisms are required in the peripheral immune system to maintain a safe T cell repertoire in healthy individuals. Peripheral tolerance is the term applied to these naturally arising mechanisms that lead to anergy, deletion or suppression of self-reactive T cells which escaped from thymus deletional processes. Starting from this evidence, researchers have sought to promote these mechanisms to obtain peripheral tolerance to alloantigens.

Various strategies have been explored to achieve peripheral tolerance in experimental protocols including: 1. targeting all peripheral T cells independently from their specificity or activation state (depleting protocols), 2. inhibiting T cell activation by

blocking or modifying costimulatory signals (costimulatory blockade, manipulation of dendritic cells), and 3. harnessing activated T cells by $CD4^+CD25^+$ antigen-specific regulatory T cells (Treg) [62].

- Lymphocyte depletion

The development of monoclonal antibodies (mAbs) has prompted studies with various lymphocyte-depleting protocols in rodents, non-human primate models, as well as in clinical transplantation, in order to prevent acute rejection and possibly to promote tolerance [63]. In various animal models, anti-T cell antibodies, given at the time of transplantation (induction therapy), were used either alone or in combination with other strategies that aim to limit clonal expansion of effector T cells. Cell-depleting approaches result in a profound reduction of circulating leucocytes capable of mounting an alloresponse at the time when the allograft is already susceptible to inflammatory damage following the ischaemia/reperfusion injury [64, 65]. Thereafter, lymphocytes will gradually repopulate the host weeks to months later when the innate immune response has resumed and the allograft is more quiescent.

Depletion strategies have been extensively studied in non-human primate transplantation models. In these studies, encouraging results were obtained using anti-CD3-immunotoxin (monoclonal anti-Rhesus CD3 antibody with a modified diphtheria toxin) alone [66], or in combination with deoxyspergualin (a monocyte inhibitor) [67] or sirolimus [68]. However in these models, despite profound peritransplant T cell depletion, consistent transplantation tolerance was not achieved with monotherapy as most treated animals eventually lost their grafts through chronic rejection [69]. Notwithstanding, this formed the basis for clinical attempts to minimize maintenance

immunosuppression after the induction of lymphocyte depletion in organ transplant recipients. This approach was attempted in 28 kidney transplant patients receiving TLI and anti-thymocyte globulin as induction and low-dose steroid as the sole maintenance immunosuppression. In three patients, immunosuppressive therapy was successfully withdrawn, suggesting that lymphocyte depletion might promote tolerance also in the clinical setting [61]. Notably, one of these patients was still off immunosuppression up to 12 years after transplant. However, the potential complications of TLI are not acceptable for routine transplantations, thus polyclonal or monoclonal antibodies were thereafter advocated as safer tools to obtain T cell depletion. Paragraph 3 will discuss attempts to transfer lymphocyte depletion in the clinical setting.

- Costimulatory blockade

Costimulation signalling is required for full T cell activation and differentiation of naïve T cells into polarized effector T cells. In the absence of an appropriate second costimulatory signal, partially activated T cells either become hyporesponsive to specific T cell receptor signals (donor-specific anergy) or die by apoptosis [70]. Overall, by inhibiting T cell activation rather than eliminating all T cells as in depleting protocols, this strategy might more selectively target effector T cells and thus spare other potentially beneficial T cell subpopulations, such as those with immune regulatory properties [71].

The CD154:CD40 pathway is of crucial importance in effective antigen presentation. CD154 (CD40L) is expressed on T cells, B cells, eosinophils, natural killer (NK) cells, platelets and dendritic cells, whereas CD40 is mainly expressed on dendritic cells, macrophages and endothelial cells and its ligation upregulates the expression of CD80

and MHC molecules [72]. Blockade of the CD154:CD40 pathway using MR1, an anti-CD40L mAb [73] which, besides blocking signal 2 may also have a cytotoxic activity towards activated T cells [74], was able to induce a condition of tolerance in a skin transplant model in the mouse. Various clones of the anti-CD154 mAb have been used in monotherapy in non-human primate models, resulting in long-term acceptance of renal, heart and islet allografts. However, allogeneic response was not fully prevented in these experiments, resulting in cellular infiltrates in the biopsies of long-term surviving allografts and eventual graft loss [75-77].

Excellent outcomes were observed first in small animal models using CTLA-4 Ig, a fusion protein with specificity for CD80/86 expressed on antigen presenting cells (APC) [78]. CTLA-4 Ig was also used and was described to prolong pancreatic islet survival [79] in non-human primates and, when used in combination with anti-CD154, to induce indefinite acceptance of renal and heart allografts, while allowing prolonged skin graft survival [66, 80].

Blocking the CD28 ligands CD80 (B7–1) and CD86 (B7–2) has been also attemped. Monoclonal antibodies targeting these costimulatory molecules, when used in monotherapy failed to significantly prolong renal allograft survival in non-human primate models. However, combined blockade of CD80 and CD86 led to prolonged survival in models of renal transplant in non-human primates, though this did not result in tolerance, as rejection occurred after therapy withdrawal [74, 81]. Two fusion proteins, abatacept and belatacept, have been recently developed to bind the ligands for CD28, the B7 molecules CD80 and CD86 [82]. A recent randomized clinical trial tested belatacept in renal transplantation showing promising results for its use as an immunosuppressive agent [83]. Although designed to study its efficacy in preventing

rejection compared with cyclosporine and not to address tolerance, this early study suggests that co-stimulation blockade will have a major role in future tolerance strategies [43].

- *Regulatory T cells*

In the past decade, it has been become increasingly clear that T cells capable of actively suppressing immune responses are at least in part responsible for the maintenance of peripheral tolerance toward self antigens [84]. Moreover, in both rodents and humans, there is an emerging consensus that immunoregulatory activity of these cells may be instrumental also for the induction and maintenance of tolerance toward alloantigens in the transplant setting [85].

The phenomenon of T cell-mediated regulation in transplantation tolerance is not new, but during the last years a number of interesting findings have brought it back into the limelight. Harnessing the capability of these suppressor cells to regulate immune responses to not only self molecules but also to foreign antigens may have an impact in the transplant setting. Indeed, the ability of these regulatory T cells (Treg) to induce unresponsiveness to alloantigens *in vivo*, in the absence of chronic immunosuppression, may inhibit the immune-mediated processes that lead to long-term graft failure [86].

Several subsets of Treg with distinct phenotypes and mechanisms of action have now been identified. They constitute a network of heterogeneous $CD4^+$ [87, 88] or $CD8^+$ [89, 90] T cell subsets and other minor T cell populations such as nonpolymorphic CD1d-responsive natural killer T cells [91, 92].

In both humans and rodents the best characterized population of Treg is the subset coexpressing CD4 and CD25 (IL-2R α chain) antigens. $CD4^+CD25^+$ Treg are defined as

'naturally occurring' or 'innate' since they arise during thymic ontogeny, selected as a result of relatively high-affinity interactions with self-peptide/MHC complexes [93]. In non-autoimmune-prone mice, elimination of $CD4^+CD25^+$ Treg, by a thymectomy carried out at day 3 of age, induced the onset of a polyautoimmune syndrome [87]. Importantly, adoptive transfer of $CD4^+CD25^+$ T cells from normal mice to thymectomized animals protected from autoimmunity [87].

In addition to their role in maintaining self-tolerance and preventing autoimmune diseases [87], $CD4^+CD25^+$ Treg play a role in preventing allograft rejection, as demonstrated in many animal models of transplant tolerance induction [94]. In a model of renal transplant tolerance by donor PBMC infusion in the rat, $CD4^+CD25^+$ Treg accumulating in tolerized kidney grafts were instrumental to the prevention of acute rejection [95].

Moreover, it has been demonstrated that $CD4^+CD25^+$ Treg with the capacity to prevent skin allograft rejection can be generated in mice by pre-treatment with donor alloantigen under the cover of non-depleting anti-CD4 therapy [96]. $CD4^+CD25^+$ Treg isolated from the spleens of these tolerant mice are donor-specific and can transfer tolerance to a naïve recipient [97]. Of great interest, evidence recently came out showing that such Treg are generated in the periphery from $CD4^+CD25^-$ precursors in a pathway distinct to that by which naturally occurring $CD4^+CD25^+$ Treg develop [98].

The main mechanism of suppression by $CD4^+CD25^+$ Treg seems to be the inhibition of IL-2 production by responder T cells [99]. Interestingly, $CD4^+CD25^+$ Treg have been shown to constitutively express CTLA4 (CD152) in both mice and humans. Fallarino *et al.* have attributed a key role to CTLA4, providing evidence that mouse $CD4^+CD25^+$ Treg can deactivate immunostimulatory function of APCs through CTLA4 engagement

of B7 molecule [100]. Thus, $CD4^+CD25^+$ Treg can exert their suppressive activity either by a direct contact with T cells or indirectly through modulation of APC function.

Since activated effector $CD4^+$ T cells also transiently express CD25, researchers looked for other phenotypic markers for identifying $CD4^+CD25^+$ Treg. That mice carrying the X-linked *scurfy* mutation in FOXP3 gene display multi-organ autoimmune disease and lack conventional $CD4^+CD25^+$ Treg [101, 102] have focused the attention on FOXP3 as a specific marker of Treg in mice. In mice, FOXP3 has been shown to be expressed exclusively in $CD4^+CD25^+$ Treg and is not induced upon activation of $CD25^-$ T cells. In addition, transfection with FOXP3 converts naïve $CD4^+CD25^-$ T cells into Treg [103]. Of particular interest, Walker et al. have shown that in humans activation of $CD4^+CD25^-$ T cells results in the generation of two populations of cells, effector $CD4^+CD25^+$ and regulatory $CD4^+CD25^+$ T cells, with expression of FOXP3 confined to the regulatory cell subpopulation [104].

$CD4^+CD25^+$ T cells expressing FOXP3 are therefore a well characterized Treg population, whose activity may play a crucial role also in tolerance induction in clinical transplantation.

Other cells with immune regulatory properties have been described. Among them, a population expressing the $CD8^+CD28^-$ phenotype has been reported to be associated to lower rates of rejection and an increased likelihood of being weaned effectively from immunosuppression in kidney and liver transplant recipients [105]. This suggests that mechanisms of peripheral tolerance are redundant and that our knowledge of them is still extremely limited.

3. Tolerance induction through lymphocyte depletion in the clinical setting

Despite the huge number of available strategies to induce tolerance in rodent models of transplantation, only few of them have been successfully transferred to nonhuman primates and, even fewer, to transplant patients. As discussed above, two recent reports showed that induction of chimerism through combined bone marrow transplantation may promote tolerance in humans [59, 60]. However, potential toxicity of the induction protocol and the complexity of the technique still make this approach hardly transferred to the clinical practice, at least not to significant numbers of patients.

So far, the pro-tolerogenic strategy that provided the most reproducible results in kidney transplant patients is lymphocyte depletion at the time of engraftment. Although this approach promotes true tolerance in only a minority of patients, it effectively allows the prevention of rejection with lower than standard amounts of chronic immunosuppression in most of the cases [106].

Theoretically, lymphocyte depletion removes effector T lymphocytes from the circulation at the time of ischemia-reperfusion injury and allows the graft to heal preventing a detrimental inflammatory response. During immune reconstitution, emergence of T cells with memory phenotype has been described (homeostatic proliferation), which might potentially represent a hurdle to the establishment of tolerance, due to their low threshold for activation [106]. However, use of compounds promoting Treg expansion during this phase might overcome this effect, thus allowing peripheral tolerance mechanisms to prevail.

Homeostatic proliferation

The peripheral T cell pool is composed of a large and heterogeneous repertoire of naïve and memory T cells capable of recognizing both foreign- and self-antigens [107]. This pool of lymphocytes is tightly regulated by homeostatic mechanisms that serve to control the numbers of T lymphocytes in circulation and the different subpopulations (*i.e.* $CD4^+$ versus $CD8^+$, naïve versus memory). This is instrumental in ensuring that the organism has continued diversity of naïve T cells able to respond to random antigenic challenges while preserving immunological memory to microbial pathogens formerly encountered [107].

Recent evidence suggests that the set point for homeostatic equilibrium involves an external mechanism of quorum sensing, which the T cell 'interprets' as a measure of available or free 'space'. Indeed, T cells seem to be under severe pressure to fill this space as evidenced by their robust expansion even in the absence of antigen soon after adoptive transfer into T cell-deficient syngeneic recipients: this is what is meant by homeostatic proliferation [108]. The same thing also happens after the induction of lymphocyte depletion.

The key hallmark of homeostatic proliferation is the induction of naïve T cells to express conventional memory T cell markers and to differentiate into a memory-like state [109, 110]. In particular, L-selectin (CD62L) which is preferentially expressed on naïve T cells, as it is necessary for entry into lymph nodes, is progressively lost as cells undergo homeostatic proliferation. In parallel with the loss in CD62L is the upregulation of CD44, a molecule whose expression is required for adhesion and entrance into peripheral tissues. Functionally, these cells are similar to memory cells as their requirement for CD28 costimulation is diminished, thus they have a lower

activation threshold [111]. Furthermore, the rapidity and magnitude of effector responses following activation, such as cytokine production, cytolytic activity and proliferative capacity and kinetics, are also greatly enhanced [109, 110]. Thus, the emergence of these cells after lymphocyte depletion might also represent a hurdle to the establishment of tolerance [112].

However, during immune reconstitution in individuals with cancer who did or did not receive IL-2 therapy, $CD4^{+}CD25^{high}$ cells also underwent homeostatic peripheral expansion during immune reconstitution, and in lymphopenic individuals receiving IL-2, the Treg cell compartment was similarly markedly increased. Along this line, mouse studies showed that IL-2 therapy induced expansion of existent Treg cells in normal hosts, and IL-2-induced Treg cell expansion was further augmented by lymphopenia [112]. Thus, the success of tolerogenic strategies with lymphocyte depletion is closely related to the equilibrium between memory and regulatory T cells.

Looking for the ideal maintenance immunosuppression after lymphocyte depletion: the effect of different drugs on Treg activity

Regulatory T cells have long been implicated in transplant tolerance. Thus, to promote tolerance after lymphocyte depletion, it would be an obvious benefit to promote the 'outgrowth' of Tregs compared to non-regulatory T cells after T cell depletion.

Intriguingly, recently evidence came out showing that different immunosuppressive compounds may exert different promoting or inhibiting effect on Tregs [113]. Finding the right combinations of immunosuppression after lymphocyte depletion might therefore identify the best maintenance regimens to use after induction with depleting agents.

Of note, evidence has been provided that sirolimus and cyclosporine, the two most widely used immunosuppressants in organ transplantation, may exert opposite effects on Treg number and function. Indeed, sirolimus seems to promote the expansion of functional Treg, whereas cyclosporine might have an inhibitory effect [114].

In particular, cyclosporin A (CsA) is a potent inhibitor of the phosphatase, calcineurin, which is essential for T-cell activation. By inhibiting calcineurin, it suppresses the production of IL-2 and related cytokines through the prevention of downstream activation of the transcription factor, nuclear factor of activated T cells (NFAT) [113]. Few *in vitro* studies described the effect of CsA on Treg. Baan et al. showed that in a mixed leukocyte reaction, the induction of FOXP3 mRNA was inhibited by CsA [115]. This was confirmed by authors of other studies, who observed decreased FOXP3 mRNA and protein [114] and a loss of the highly suppressive $CD27^+$ Treg subset in cultures containing CsA [116]. The authors of these later studies report contradicting effects of CsA on the suppressive function of Treg, one observing no effect with human Treg and the other finding less suppression with mouse Treg [113].

There are now recent *in vivo* data that show a negative effect of CsA on Treg. Treatment of mice with CsA compromised not only the thymic generation of Treg but also resulted in a sharp reduction of Treg in peripheral immune compartments [117]. In a mouse bone marrow transplantation model, CsA administration inhibited Treg mediated suppression which was associated with reduced IL-2 production [118]. Together these data suggest that CsA is not beneficial for Treg, but is rather detrimental to their generation, survival and function.

Sirolimus exerts its effect at the level of mammalian target of rapamycin (mTOR), thereby preventing the progression from G1- to S-phase. There is *in vitro* and *in vivo*

evidence that sirolimus treatment has favourable effects on Treg. In a study with mice by Battaglia et al., $CD4^+CD25^+FOXP3^+$ Treg expanded ex vivo in the presence of sirolimus and prevented rejection of beta-islet transplants *in vivo* [119]. Sirolimus also induces de novo expression of FOXP3 in murine alloantigen-specific T cells dose dependently, which appeared to be TGF-β1 dependent [120]. Because sirolimus can induce the expression of TGF-β1, it may be an important mechanism contributing to the development of antigen-specific Treg [113]. Interestingly, a recent study suggests that sirolimus can induce regulatory functions in conventional $CD4^+$ T cells in culture [120]. Furthermore, evidence suggests that sirolimus -conditioned dendritic cells are poor stimulators of allogenic T cells but enrich for antigen-specific Treg, which can prolong cardiac graft survival in mice [121].

Altogether, the above evidence suggest that the choice of the maintenance immunosuppressive regimen after lymphocyte depletion may be crucial for the success of this pro-tolerogenic strategy.

Different strategies to induce lymphocyte depletion in kidney transplant patients

Lymphocyte depletion using polyclonal antibody therapy has long been a part of the transplant immunosuppressive armamentarium and is reserved for immunologically high-risk recipients. More recently, the use of both polyclonal and monoclonal depletion has been adopted to allow immunosuppression minimization or even to achieve donor-specific tolerance [122]. The long-term aim of this therapy is to minimize the toxicities we have come to expect with standard immunosuppression, which may be limiting long-term outcomes. This positive effect is offset by the potential toxicity of the global depletion of the recipient's lymphocytes and, in some cases, monocytes and neutrophils.

Efforts aimed at identifying effective and well-tolerated induction protocols may maximize the long-term success of kidney transplantation.

Seminal studies in nonhuman primate transplantation models showed that lymphocyte depletion by total lymphoid irradiation (TLI) prolonged graft survival and promoted donor-specific hyporesponsiveness [123]. On the basis of this background, 28 kidney transplant patients received TLI and anti-thymocyte globulin as induction therapy and low-dose steroid as the sole maintenance immunosuppression. Immunosuppressive therapy was successfully withdrawn in three patients, which suggests that lymphocyte depletion might also promote tolerance in the clinical setting [61]. Of note, one of these patients was free of any imunosuppression for more than 12 years [61]. The potential complications of TLI are not acceptable for routine transplantation, however, and polyclonal or monoclonal antibodies were therefore advocated as safer tools for obtaining T-cell depletion.

In the 1970s, the polyclonal antibody Minnesota antilymphoblast globulin was introduced in the immunosuppressive treatment of organ transplant recipients and were used for more than twenty years, together with azathioprine and steroids (and, from 1978, cyclosporine), to prevent and treat acute rejection. In spite of the increased risks of anaphylactic reactions and opportunistic infections, this experience highlighted the importance of lymphocyte depletion to improve patient and graft outcomes [123].

More recently, new formulations of polyclonal rabbit anti-thymocyte globulin have become available and have been progressively introduced in the induction protocols of most transplant centers. However, some concerns still persist in the extensive use of polyclonal antibodies, as they have been associated with a significant increased risk of opportunistic infection and lymphomas. Of note, potential immunization against non

human immunoglobulin, might induce anaphylactic reactions and prevent repeated administrations. Thus, during last decade, research has been focused on humanized monoclonal antibodies [123].

4. Campath-1H

Campath-1H is a humanized rat monoclonal antibody (rat immunoglobulin IgG2b) directed against the CD52 antigen, which is expressed on all blood mononuclear cells and also on cells lining the male reproductive tract [124]. It is a powerful cytolytic agent and has been used therapeutically in bone marrow and organ transplantation, and in several autoimmune diseases [124]. It was first used by Sir Roy Calne [125] as induction therapy for renal transplantation in 1998 and it efficiently prevented acute rejection in 13 patients who received low-dose CsA as the sole immunosuppressant. Since then, many other trials employed this antibody to induce a pro-tolerogenic state that may allow reducing doses of maintenance immunosuppression [126].

A brief historical perspective

The first Campath-1H antibodies were created from rat hybridomas in an attempt to produce antibodies that would lyse lymphocytes in the presence of human complement [124]. All these antibodies were directed against the same antigen, now known as CD52. The first antibody was a rat IgM (Campath-1M) that resulted in only a transient lymphopenia in patients with leukaemia. Thereafter, an IgG antibody (Campath-1G) was developed, which was an IgG2b antibody, produced as a switch variant of IgG2a [124]. This antibody was profoundly lytic in the presence of human complement but also produced direct lysis by antibody-dependent cellular cytotoxicity. Finally, to

prevent the development of rat globulin antibody responses in patients, the rat antibody was humanized (Campath-1H), the first such successful humanization of a clinically used monoclonal antibody [127]. Campath-1H and Campath-1G have very similar lytic activities, but Campath-1H has gradually replaced Campath-1G in clinical practice over the past 10 years. In addition, in two studies of Campath-1H antibodies used to treat acute rejection, 15 of 17 patients given the rat antibody Campath-1G exhibited a rat antiglobulin response, in contrast to none of 12 patients given the humanized antibody Campath-1H [128]. No anti-idiotype antibodies were detected, but it should be noted that repeat courses of the antibody were not given and that the patients were all receiving concurrent immunosuppression. In contrast, three of four patients with rheumatoid arthritis who received a repeat course of the antibody in the absence of other immunosuppression developed an anti-idiotype response [126].

The function of CD52 is currently unknown. It is a short glycoprotein consisting of a sequence of only 12 amino acids. It is attached to the outer layer of the cell membrane by a glycosyl phosphatidylinositol lipid anchor. The CD52 antigen is one of the most abundant antigens on the surface of lymphocytes, accounting for approximately 5% of the surface antigens [124]. This probably explains in part the profound and long-lasting lymphopenia produced after the administration of one or two doses of the antibody.

Campath-1H as induction agent in kidney transplantation

Campath-1H was first used as an induction agent by Calne et al. [125] in 1998 in 13 renal transplant recipients who received low-dose cyclosporine alone as maintenance therapy. At the time of publication, patient and graft survival rates were 100% and there were two episodes of acute rejection, with a follow-up of 6-12 months. Azathioprine

and prednisolone were added to one patient's immunosuppressive regimen to treat rejection. The 5-year results of the initial series were published along with those of another 20 patients who were subsequently entered in this pilot trial (total of 33 patients) [129]. They found no significant difference in graft or patient survival or acute rejection rates in a retrospective contemporaneously controlled comparison with the findings in 66 patients who underwent kidney transplantation in the same unit during the same period and were treated with triple therapy (cyclosporine, azathioprine, and prednisolone). This led to hypothesize that Campath-1H, by reducing the need of maintenance immunosuppression (and related toxicity) to prevent acute rejection, might actually provide better outcomes in the longer term. However, seven patients in the control group were in a highly sensitized condition and received induction therapy with thymoglobulin. Thus, the higher immunological risk of patients in the control group might have lead to overestimate the beneficial effects of patients who received Campath-1H induction.

In light of the limitations of this study, Calne et al recently coordinated a randomized, controlled, prospective trial comparing the 12 month outcomes of 65 patients who received Campath-1H induction and delayed tacrolimus monotherapy versus those of 66 patients on tacrolimus, MMF and steroids without induction. Results were very similar in the two groups, apart for an higher incidence of CMV infections in Campath-1H treated patients [129].

Ciancio *et al.* [130] designed a well planned three-arm trial with 30 patients in each arm in which compared induction with Thymoglobulin, Campath-1H, and daclizumab (an anti IL-2 receptor antagonist). All patients received maintenance immunosuppression with tacrolimus, mycophenolate mofetil (MMF), and steroids, but the Campath-1H

group received half the dose of tacrolimus and no steroids after the first week. After a median follow-up of 15 months, there was no difference in patient or graft survival, acute rejection rates, or renal function, nor was there any difference in infections or incidence of diabetes or hyperlipidemia among the three groups. However, 80% of the Campath-1H patients remained steroid-free. Intriguingly, patients in the Campath-1H arm showed an higher expansion of Treg.

A number of nonrandomized retrospective studies with large numbers of patients have been reported in which Campath-1H was compared with other induction therapies in renal transplant recipients [126]. Knechtle et al. [131] compared induction with Campath-1H (n=126) to historical control groups treated with anti-CD25 antibody (basiliximab; n=799), Thymoglobulin (n=160), and other induction therapies, such as OKT3 or antithymocyte globulin (n=156). For maintenance immunosuppression, all groups received a calcineurin inhibitor and MMF. Prednisone was used in all groups except the group that received Campath-1H. There was a marginal reduction in the incidence of biopsy-proven acute rejection (P=0.037) and a better graft survival (P=0.0159) in the Campath-1H group. Also, when looking at the subgroup of patients who experienced delayed graft function, there was significantly less acute rejection (P=0.0096) and a significant improvement in graft survival (P=.0119) in the Campath-1H group. There was no significant difference in patient survival nor in the incidence of infection and malignancy among the three groups.

Before this study, the same group [132] performed a pilot trial with Campath-1H induction and sirolimus monotherapy in 29 patients. Thirteen patients had an acute rejection, which in six cases was of the humoral type. However, the 3-year results showed graft and patient survival rates of 96% and 100%, respectively, with an

excellent graft function. In another pilot study of Campath-1H induction with MMF and sirolimus in 22 kidney transplant recipients [133], there were eight acute rejections (36%) with leukopenia and possible pulmonary toxicity, leading the authors to suggest that initial use of a calcineurin inhibitor might be necessary with Campath-1H induction.

Shapiro et al. [134] compared Campath-1H induction (n=90) versus historic control patients treated with Thymoglobulin induction (n=101) and a non-induction therapy group (n=152). In the control group without induction therapy, the maintenance immunosuppression was tacrolimus, prednisolone, and usually a third agent (MMF or sirolimus). Both induction therapy groups received tacrolimus as maintenance monotherapy. After 3-4 months, spaced weaning of the tacrolimus was attempted in the induction therapy groups. There was no significant difference in overall graft or patient survival, but when looking at the subgroup of living-donor grafts, graft survival was significantly better for Campath-1H and Thymoglobulin compared with the control group without induction therapy (P=0.037). The acute rejection rate was similar in the Campath-1H and control groups, which was better than that in the Thymoglobulin group.

In an attempt to induce donor allograft tolerance, Campath-1H was also used as induction therapy alone with no maintenance therapy in seven nonsensitized recipients of living-donor transplant kidneys at the NIH Renal Transplant Center [135]. All seven patients developed early rejection within the first month, requiring initiation of maintenance immunosuppression, but all rejection episodes were successfully treated. More recently, the same authors [135] treated a further five recipients of a living-donor kidney with Campath-1H and a brief course of deoxyspergualin, which was added to the

treatment regimen with the aim of preventing the early macrophage and monocyte infiltration observed in the patients treated earlier [136]. However, all patients exhibited a reversible rejection similar to the aforementioned group and rejection was preceded by or associated with marked increases in several chemokine transcripts. Therefore, used in this protocol, Campath-1H was unable to produce tolerance.

Recently, an analysis of Organ Procurement and Transplantation Network/United Network for Organ Sharing database compared the outcomes at 2 years of deceased donor kidney recipients treated with Campath-1H (n=690), thymoglobulin (n=4,930), interleukin-2 receptor antagonist (n=4,378), or without induction [137]. Patients on Campath-1H therapy experienced less acute rejection during the initial hospitalization comparing to the other groups, but this finding was not sustained 6 and 12 months after transplant, when the rejection-free survival of Campath-1H patients was significantly lower than the one of patients who received other induction agents or without induction. Despite this increased acute rejection risk, graft survival did not significantly differ among various patients groups. The increased incidence of late rejections in Campath-1H-treated patients might reflect the attempts to reduce immunosuppressive therapy among patients in this group. Importantly, among recipients of Campath-1H induction, rejection-free survival and graft survival were significantly higher when maintenance immunosuppression included calcineurin inhibitors.

Campath-1H safety profile in kidney transplantation

The profound and long-lasting lymphocyte depletion induced by Campath-1H raised concerns about its safety profile, especially in the long-term. Notwithstanding, it is in general well tolerated and the severity of potential side effects is mild.

- Cytokine release syndrome

As with all antibody treatments, Campath-1H infusion may induce a cytokine release syndrome characterized by fever and hypotension. It is generally modest and normally controlled with an intravenous bolus injection of 1 g of methylprednisolone before administration of the antibody. Recently, however, a study showed that, in kidney-pancreas transplantation, subcutaneous administration of Campath-1H avoids this first-dose reaction while achieving a similar lymphocyte depletion to intravenous administration [138]. Prevention of infusion-associated hypotension is particularly important in pancreas transplantation where venous thrombosis is a major problem.

- Infection

Despite the profound and long-lasting CD4 T cell depletion for 2-3 years produced by one or two doses of Campath-1H, there has been a surprising lack of serious infection in nearly all studies reported. Silveira and colleagues [139] examined a cohort of 449 consecutive transplant recipients who received Campath-1H to determine the incidence of bloodstream infections, which might be expected to have an increased incidence in patients in a CD4-depleted state, as seen in patients with AIDS, for example. No increased risk was noted. Similarly, a low incidence of infection was noted in another small study in comparison with a historical control group [140], and indeed this has been a feature of all the reports described earlier.

- *Autoimmune disease*

An interesting observation made in the long-term study of Watson et al. [129] was the occurrence of an autoimmune disorder in two patients who had received Campath-1H, one with autoimmune hypothyroidism and one with autoimmune hemolytic anemia. This is relevant bearing in mind that, of 27 patients with multiple sclerosis treated with Campath-1H, nine developed autoimmune hyperthyroidism [141]. Recently, also the case of a kidney transplant recipient who developed autoimmune thyroiditis four years after Campath-1H induction has been reported [142]. Thus, although rare in the setting of organ transplantation, increased risk of autoimmune disease should be considered when facing transplant patients receiving Campath-1H induction. This risk might be even higher considering that, in transplant patients, this adverse event might have been underreported.

- *Coagulopathy*

Campath-1H administration has been also associated both with disseminated intravascular coagulation (DIC) in a patient with bone marrow transplantation and with massive bleeding in a kidney transplant recipient. In both cases, the adverse event followed the first Campath-1H administration. No clear mechanism has been advocated to explain how CD52 antibody may affect coagulation.

Do patients with Campath-1H induction reject their grafts in spite of the absence of circulating T lymphocytes?

Unexpectedly, the aforementioned clinical experiences showed that acute rejection does occur in renal transplant recipients who undergo induction therapy with Campath-1H, despite the profound lymphocyte depletion. However, the histologic pattern may be different than the one observed with conventional immunosuppression. Comparing biopsies of renal transplant recipients with acute rejection who did or did not receive Campath-1H induction, Zhang *et al.* showed that Campath-1H induction was associated with acute rejections with a majority of the cells (up to 95%) being monocytes and a minority T lymphocytes [143]. Conversely, in renal specimens from patients who did not receive Campath-1H induction, monocytes were mixed with many other inflammatory cells including T lymphocytes, eosinophils and neutrophils [143]. This is consistent with the fact that Campath-1H can severely deplete peripheral T lymphocytes to minimal levels, whereas its effect on monocytes is much milder. These findings confirm those previously observed by Kirk *et al.* who tried to use Campath-1H alone [135] or followed by a deoxyspergualin [136] in two small series of kidney transplant recipients . All patients treated with these protocols experienced an acute rejection episode that required the introduction of maintenance immunosuppression. Intriguingly, all the rejection episodes were mainly mediated by infiltrating monocytes. The authors suggested that monocyte and macrophage lineage cells resistant to Campath-1H release cytokines in the graft, perhaps in an attempt to recruit effector T cells and, in doing so, they induce renal dysfunction. Importantly, all the acute rejections responded to typical rescue therapy. After reversal of acute rejection, the clinical scenario is most consistent with immunologic indifference rather than tolerance. That is to say that the healed allograft does not induce sufficient chemotactic signals to attract a significant

inflammatory infiltrate, thus acute rejection can be prevented also with small amounts of maintenance immunosuppression.

When Campath-1H induction was followed by maintenance monotherapy with sirolimus, an increased incidence of acute rejections of the humoral type was observed. In a small prospective study, five out of 29 patients treated with this immunosuppressive strategy experienced an acute humoral rejection that in one case resulted in loss of the graft [132]. However, in the trial by Kaufman et al. [144] of tacrolimus and MMF, there were no episodes of acute rejection that were humoral in origin or macrophage-mediated. In a number of other studies, no histologic details were given other than a statement that rejection was graded based on Banff criteria. This suggests that, in the presence of a calcineurin inhibitor after induction with Campath-1H, humoral rejection is uncommon.

Recently, Gallon *et al.* [145] compared kidney graft biopsies during an acute rejection episode of 12 patients who received Campath-1H induction and MMF and tacrolimus as maintenance therapy with those of a control group of transplant patients who did not receive induction therapy. They confirmed previous data showing that monocyte infiltration is higher among patients who received Campath-1H induction, but they also pointed out that T cells in the rejecting grafts displayed more frequently a memory phenotype. As previously discussed, these cells might indeed be more resistant to Campath-1H, thus potentially representing a hurdle to the induction of tolerance.

5. Tools to monitor the immune response in organ transplant recipients

Monitoring immune reactivity of transplant patients is instrumental for understanding the mechanisms underlying tolerance and may aid in the design of strategies for the induction of tolerance in transplantation. Moreover, identification of immunological

tolerance would allow the partial or complete cessation of immunosuppressants in selected patients, a highly attractive goal, given the morbidity and mortality associated with long-term administration of such therapy. However, despite the pressing need to develop tolerance assays, to date, no one has been shown to predict accurately the development or presence of donor-specific tolerance after transplantation. Many promising candidate assays measure the presence of anti-donor responses or proinflammatory responses *ex vivo*, usually in peripheral blood lymphocytes.

Immune monitoring assays that currently are in development can be divided broadly into two major categories: donor antigen specific and antigen non-specific. Donor antigen–specific assays measure the response of T and B cells to specific donor antigens, whereas antigen-nonspecific assays for the most part determine the phenotype of surface markers or functional state of cells with the goal of identifying a pattern that is associated with a particular clinical status [146].

Antigen-specific assays for monitoring transplantation immunity and tolerance

Strict definitions of transplantation tolerance include impaired responses to donor antigens with maintenance of immune responsiveness to third-party and non-donor antigens. Therefore, assays that evaluate donor-specific responses of recipient lymphocytes are likely to be informative in transplantation. Assays of T cell reactivity that reflect antigen-specific responses include the mixed leukocyte reaction (MLR), the limiting dilution (LDA), the enzyme-linked immunospot (ELISPOT) and the trans vivo delayed-type hypersensitivity (DTH) assays [146].

Mixed Lymphocyte Reaction (MLR)

Mixed lymphocyte reaction (MLR) represents one of the first assays developed to measure proliferative response of lymphocytes towards HLA mismatched cells. In its classical form, peripheral blood lymphocytes from two individuals are mixed together in tissue culture for several days; lymphocytes from incompatible individuals will stimulate each other to proliferate significantly (measured by tritiated thymidine uptake) whereas those from compatible individuals will not; in the one-way MLR test, the lymphocytes from one of the individuals are inactivated thereby allowing only the untreated remaining population of cells to proliferate in response to foreign histocompatibility antigens [147].

In kidney transplant recipients, donor-specific hyporesponsiveness assessed by MLR at 3 and 6 months after transplantation was associated with better graft outcome at 1 year [148]. A recent study in pediatric kidney transplant recipients showed that donor-specific hyporesponsiveness was associated with improved graft survival also at 3 years and with lower incidente of CAN [149]. Moreover, these data suggest that although downregulation of donor-specific reactivity might not be a prerequisite for stable graft function it could help identifying recipients who require less immunosuppression [149].

Limiting dilution assays

Limiting dilution analysis (LDA) is a method for determining the frequency of defined clones of lymphocytes responding to a specific antigen or with a particular effector function [150]. The technique consists of setting up multiple replicates of graded dilutions of responder cells (usually patients' unselected peripheral blood lymphocytes or purified populations of $CD4^+$ or $CD8^+$ cells) in wells containing a non-limiting stimulus (e.g. donor stimulator cells). The readout from a particular well is only

considered positive if the measure chosen exceeds the mean of controls (cultures lacking responder cells) by a factor of three or more. The number of 'negative' wells at each dilution of responder cells is determined. As the concentration of the responder cells increases, the proportion of 'negative' wells will tend to be less; the relation between the number of negative cultures and the mean number of precursors can be plotted and a frequency obtained [151].

LDA has been effectively used to predict graft-versus-host disease (GVHD) and survival of bone marrow transplantation [152, 153]. In solid organ transplants, the data is less abundant, and conflicting data have been reported in the ability of CTLp measurement to predict rejection [154-156].

Enzyme-linked immunospot (ELISPOT)

ELISPOT assay is a hybrid that combines features of a MLR and an ELISA assay in that responder/recipient T cells are cultured with inactivated stimulator/donor or third-party cells in tissue culture plates that are coated with an antibody that is specific for the cytokine of interest (many cytokines have been studied, including IFN-γ, IL-2, IL-4, IL-5, and IL-10). After a fairly brief culture period, the cells are washed away and the bound cytokine is detected, using labeled secondary antibodies and an automated plate reader. Each spot that is detected represents a cell that had been primed to the stimulating antigen(s) *in vivo* (effector or memory T cells). Thus, this assay measures the frequency of previously activated or memory T cells that respond to donor antigens by producing a selected cytokine rather than the total amount of cytokine that is produced and secreted into supernatants (as measured using an ELISA). This is an important advantage because cytokines are captured immediately upon secretion from

cells, whereas cytokines that are secreted in supernatants may be subject to breakdown or dilution or may be used up by other cells [146].

The frequency of IFNγ-producing cells detected by ELISPOT has been shown to be low in stable recipients of renal allografts and significantly increased in those recipients who experienced rejection [157]. In a follow-up study, the mean frequency of T cells primed to donor antigens at 6 months was shown to correlate with serum creatinine at 6 and 12 months independently of acute cellular rejection, delayed graft function, or the recipient's panel-reactive antibody [158]. The frequency of donor-reactive cells, primed through the direct or indirect pathway, was also shown to be increased in kidney transplant recipients with chronic allograft nephropathy [159].

Transvivo delayed-type hypersensitivity (DTH) assay

In this assay, human peripheral blood mononuclear cells are injected with specific antigens into either the footpad or the pinna of immunodeficient mice, and the magnitude of the resultant swelling after 24 h is taken as an index of the reactivity of these cells to that antigen [31]. VanBuskirk AM, et al. described four transplant recipients in whom all immunosuppression had been discontinued [160]. Three of these patients, who had prolonged drug-free graft survival, were shown to have alloantigen-specific hyporesponsiveness in the trans vivo DTH assay. By contrast, the fourth patient, who had previously displayed, but lost, operational tolerance, had a strong alloantigen-specific trans vivo DTH response [160].

Theoretically, the trans vivo DTH may be useful as a tool for identifying tolerant transplant recipients. However, because of the need for mice and that the assay is relatively cumbersome, the utility of this assay for routine clinical immune monitoring

is uncertain. Nevertheless, this assay also is being evaluated in larger, multicenter trials and may be helpful mainly as a research tool.

Non antigen-specific assays for monitoring transplantation immunity and tolerance

A number of non–antigen-specific assays that may be useful for post-transplantation monitoring of the recipient immune response have been described. Such assays include phenotyping of recipient cells, quantifying the response of recipient T cells to polyclonal stimulation *in vitro* and quantification of gene expression.

Phenotyping of recipient immune cells

Regulatory cells have been shown to be important for controlling immune responses in a number of pathogenic disease processes as well as after transplantation. A clear relationship between the presence and activity of Treg and clinical transplant outcomes has not yet been clearly shown. However, the presence of Treg might theoretically represent a marker of immunoregulation in stable transplant patients and phenotypical analysis of lymphocytes isolated from transplant recipients could help to identify patients who may be given a lower immunosuppression.

Other markers of leukocyte activation might be useful in defining the level of immune reactivity, thus helping in shaping immunosuppressive therapy.

T cell responses to polyclonal, non–antigen-specific stimulation

Beside assays evaluating T cell activation in response to allogeneic donor MHC molecules, it is possible to quantify T cell aspecific response toward a polyclonal stimulus. To this purpose, different assays have been developed. One of the most

common consists of stimulating blood lymphocytes with phytohemagglutinin in ELISPOT plates. The number of spots specific for a target cytokine (usually IFN-γ) after a short culture are a measure of lymphocyte reactivity [146].

Assays to quantify gene expression

Extensive studies that have been conducted using animal transplant models have used PCR analysis of gene expression to show associations between the expression of certain genes and the nature of the recipient anti-donor immune response. Recently, the measurement of FOXP3 mRNA in urine was reported to correlate with the outcome of acute rejection after renal transplantation, with increased expression of FOXP3 associated with a greater likelihood of reversal and improved graft survival [161].

6. *In vitro* alloreactivity of transplant patients

Evaluating *in vitro* alloreactivity of transplant patients represents a major tool for understanding mechanisms at the basis of allo immune response and for identifying potential ways to promote tolerance. To this purpose, patients with stable graft function focused transplant immunologists' interest the most.

Interestingly, a large fraction of these patients show a low *in vitro* alloreactivity. Different, yet not mutually exclusive, mechanisms of donor-specific hyporesponsiveness have been proposed, including regulation and anergy.

The first clinical efforts to study the role and the relevance of $CD4^+CD25^+$ Treg in the regulation of alloimmune responses in transplant patients has only recently emerged [162, 163]. The frequency and functional profile of circulating $CD4^+CD25^+$ T cells have been evaluated in 10 lung transplant recipients with stable clinical condition and in 11

with chronic rejection [164]. The frequency of $CD4^+CD25^+$ T cells were significantly higher in stable transplant patients as compared with that found in patients with chronic rejection. In addition, functional evaluation of these cells demonstrated their regulatory profile: they were hyporesponsive to conventional T cell stimuli and suppressed the proliferation of $CD4^+CD25^-$ T cells [164].

To better clarify the function of $CD4^+CD25^+$ Treg in clinical transplantation, their role in regulating both the direct and indirect pathway has been evaluated. Salama *et al.* [162] reported on twenty-three renal transplant patients, grouped into two cohorts with or without an history of acute rejection. These patients were chosen on the basis of their low reactivity to the mismatched donor-derived HLA-DR antigen. By employing ELISPOT assay, the authors were able to detect significant increase in the frequency of IFN-γ-producing cells stimulated by donor-derived mismatched HLA-DR peptides, after depletion of the $CD25^+$ subset. This increase was alloantigen-specific, as the response to recall mumps antigen was unaffected by CD25 depletion. Notably, this frequency increase was associated with the history of graft rejection, and the initial status of alloresponses toward the mismatched alloantigen *in vitro*. Conversely, Game *et al.* [165] failed to detect any changes in the direct alloreactivitiy specific to donor-type alloantigens after CD25 depletion. By screening twelve stable renal transplant patients, the authors measured the effects of $CD4^+CD25^+$ T cell depletion on alloresponses in the direct pathway by a Limiting Dilution Assay (LDA), as well as by ELISPOT for IFN-γ. In 11 out of 12 patients, no significant increases were detected in the frequency of donor-specific T cells after depletion of the $CD25^+$ subset. In one case, the increase occurred in both donor- and third party-reactive T cells. Thus, they concluded that $CD4^+CD25^+$ T cells are not the major regulators responsible for donor-specific direct T

cell hyporesponsiveness. This conclusion is supportive of their previous experimental results showing anergy as one of the mechanisms of hyporesponsiveness of anti-donor T cells in the direct pathway [162]. Subsequent data confirmed that hyporesponsiveness toward donor antigens in organ transplant patients with stable function is, at least in part, sustained by Treg immune regulation of the indirect pathway [166, 167]. Still, the role of Treg has subsequently been reported also in the control of direct recognition pathway of alloantibodies [168]. Indeed, $CD4^+CD25^+Foxp3^+$ T cells harvested from renal transplant patients are able to suppress both indirect and direct alloproliferation *in vitro*. However, a functional analysis of circulating Treg (by depleting/reconstituting experiments) harvested from renal transplant recipients maintained on different immunosuppressive regimens showed that these cells mediate donor hyporesponse only in a subset of patients [169].

Intriguing data are also emerging about different promoting or inhibiting effects of various immunosuppressive agents on Treg number and function. Indeed, chronic immunosuppression with CsA in renal transplant patients has been associated with lower levels of circulating Treg as compared with SRL immunosuppression, possibly due to the inhibitory effect of calcineurin inhibitors on IL-2 pathways, that are required for Treg proliferation [114, 170].

Another population of T cells with regulatory effects expressing the $CD8^+CD28^-$ phenotype was associated with lower rates of rejection and an increased likelihood of being weaned effectively from immunosuppression in kidney and liver transplant recipients [105]. FOXP3 positive $CD8^+CD28^-$ T suppressor (T_S) cells are antigen specific, MHC class I-restricted, and interact directly with antigen-presenting cells (APC). T_S render antigen presenting cells tolerogenic, inducing the downregulation of

costimulatory molecules and upregulation of the inhibitory receptors, immunoglobulin-like transcripts (ILT)3 and ILT4. ILT3 and ILT4 display long cytoplasmic tails containing immunoreceptor tyrosine-based inhibitory motifs (ITIM), which mediate inhibition of cell activation by recruiting tyrosine phoshatase SHP-1. The interaction between allospecific $CD8^+CD28^-$ cells and epithelial cells is bi-directional since tolerogenic $ILT3^+ILT4^+$ epithelial cells induce the *in vitro* differentiation of $CD8^+$ into $CD8^+CD28^-$ T cells [171, 172]. So far, however, only few groups have focused on their role.

Beside Treg, a study by Lechler et al. in human renal transplant recipients indicated that T anergy plays an important role in maintaining hyporesponsiveness toward donor antigens [165]. They demonstrated that donor-specific hyporesponsiveness can be specifically reversed by *ex vivo* treatment of recipient $CD4^+$ T cells with IL-2 in stable renal transplant patients, consistent with the hypothesis that anergy may contribute to the decrease in anti-donor frequencies. Conversely, the third-party frequencies were unaffected by IL-2 stimulation, also indicating that the increase in anti-donor frequency after IL-2 is unlikely to be due to nonspecific stimulatory effect of IL-2 [165].

The finding that IL-2–driven cell division can reverse hyporesponsiveness in direct pathway T cells may have relevance to the link between systemic infections such as CMV or local infections of the urinary tract and acute rejection episodes. Conceivably, infection of the urinary tract can result in production of IL-2 locally or in the draining lymph nodes, leading to reversal of the anergic state of the allospecific T cells and consequent acute rejection.

Effect of Campath-1H induction on alloreactivity of transplant patients

Lymphocyte depletion has long been pursued as a therapeutic strategy to induce tolerance since the earliest days of transplantation. Indeed, after depletion, the emerging graft-specific T cells were thought to encounter donor antigens in a healed state and to be purportedly reinforced to become anergic. Thus, the availability of a relatively safe lymphocyte depleting antibody such as Campath-1H offered the opportunity to test this hypothesis in the clinical setting. The clinical experience accumulated so far actually confirms these pro-tolerogenic properties of peri-operative lymphocyte depletion, as it allows prevention of acute rejection with doses of maintenance immunosuppression significantly lower than the ones used in conventional regimens. However, studies aimed at evaluating the mechanisms at the basis of these immunemodulating effects are still few.

It is now clear that sensitivity of various CD52 positive cells to Campath-1H depletion is variable, with antigen-experienced memory T cells being less susceptible to depletion than naïve cells [173]. Consequently, differences in recipient's allospecific immune repertoire at transplantation can cause relative resistance or sensitivity to depletion. Recently, Trzonkowski *et al.* found that after Campath-1H induction the recovery of $CD8^+$ T cells was much faster than that of $CD4^+$ T cells [174]. Of note, repopulating $CD8^+$ T cells were mainly of immunosenescent $CD28^-CD8^+$ phenotype and were able to suppress $CD4^+$ T cell proliferation. Intriguingly, the authors hypothesize that expanded $CD28^-CD8^+$ T cells might compete for 'immune space' with $CD4^+$ T cells suppressing their proliferation and therefore delaying $CD4^+$ T-cells recovery [174]. This delay might be associated with the clinical outcome as $CD4^+$ T cells, notably $CD4^+$ T effector memory cells, were shown to be associated with rejection. These findings, combined with those indicating that lymphopenia induces some degree of general homeostatic

activation, suggest that the specific immune capabilities and requirements for T cell immunosuppression during repopulation after Campath-1H induction may vary among individuals as a function of their T cell repertoire maturity.

As for the functional profile of repopulating lymphocytes, Bloom et al. [175] compared anti-donor and third party responses of T cells isolated from patients receiving Campath-1H induction and sirolimus as maintenance monotherapy with those from patients treated with basiliximab induction and on maintenance therapy with CsA, MMF and steroids. Interestingly, they found that proliferative responses to donor antigens were equal between Campath-1H and control group, but T cells from Campath-1H patients displayed a greater response to third-party antigens suggesting that Campath-1H induction combined with sirolimus monotherapy may promote donor-specific hyporesponsiveness. As stated above, however, this immunosuppressive approach is burdened by a too high incidence of acute humoral rejections, thus the real meaning of donor-specific immunosuppression may in fact vary greatly among different patients [132].

7. Campath-1H: still unanswered questions

Most of the experience with Campath-1H in kidney transplantation comes from small, non randomized studies. However, the collective experience accumulated so far suggest that Campath-1H infusion at the time of transplantation is able to modulate the immune system response to the point that acute rejection can be prevented with lower than conventional doses of maintenance immunosuppression. Early experiences also demonstrated that Campath-1H induction alone is however not sufficient to prevent rejection without minimal doses of maintenance immunosuppression.

The best maintenance therapy after Campath-1H induction still needs to be identified. Seminal experience by Calne [125] showed that maintenance immunosuppression with low doses of cyclosporine monotherapy are effective in preventing acute rejection and similar findings were subsequently reported also with tacrolimus [144]. Conversely, sirolimus monotherapy was associated with too high an incidence of acute rejection of the humoral type [132]. When mycopheonalate mofetil was associated to sirolimus, the incidence of acute humoral rejections dramatically declined [133]. So far, however, randomized prospective studies comparing different maintenance immunosuppressive regimens after Campath-1H induction are lacking. Moreover, it would be important to investigate long-term graft histology changes in patients who received Campath-1H induction and different low-dose maintenance immunosuppressive regimens.

Importantly, different maintenance immunosuppressive agents might also exert different effects on lymphocyte phenotype and function. Indeed, data suggest that cyclosporine, by affecting IL-2 signalling, might impair the proliferative capabilities of Treg [176], whereas sirolimus seems to promote their expansion [177]. On the other hand, only cyclosporine seems to provide enough immunosuppression to inhibit memory T lymphocytes, those cells that are largely spared by Campath-1H [173].

Thus, comparing the effect of different maintenance immunosuppressive regimens after Campath-1H induction both on the phenotypic and functional characteristics of peripheral lymphocytes and on clinical outcomes might be of utmost importance.

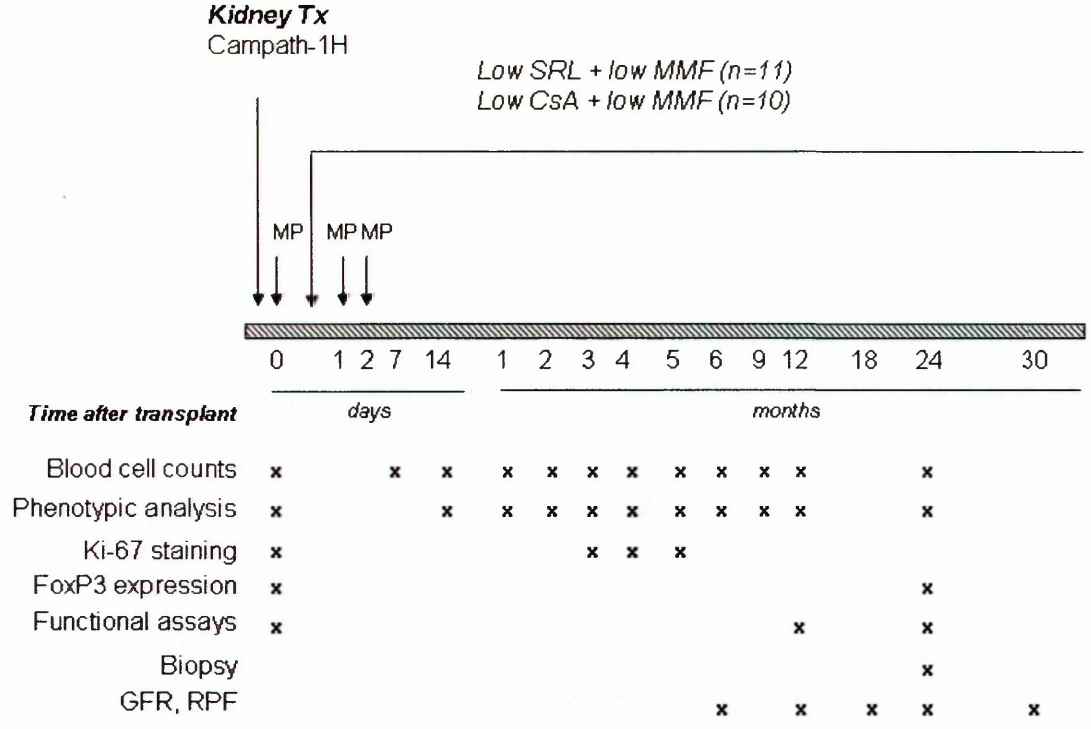
METHODS

Patients and study design

Twenty-one patients (13 men and 8 women) with end-stage renal disease who underwent primary kidney transplant were enrolled under an Ethics Committee-approved protocol at the Ospedali Riuniti Bergamo, Italy, following written informed consent. Primary cadaver (n=19) and living-related (n=2) donor renal transplant recipients were selected based on the following criteria: age 18-70 years, current PRA <10%, non-HLA identical to the donor. A negative CDC crossmatch test was required prior to transplantation. Eligible patients were allocated to one of the following two study groups according to a randomization design: Group 1 (n=11) was assigned to Campath-1H, low-dose sirolimus (SRL) and low dose Mycophenolate mofetil (MMF); group 2 (n=10) entered to a regimen with Campath-1H, low-dose Cyclosporine (CsA) and low-dose MMF (Figure 1). Randomization was performed at the Laboratory of Biostatistics of the Clinical Research Center for Rare Diseases "Aldo e Cele Daccò" of the Mario Negri Institute. Patients consented to serial monitoring of their blood leukocyte population phenotype and function. At one year after transplantation, mean circulating Treg count was four-fold higher in SRL than CsA-treated cohort. Thus, we designed an extension phase of the study. All patients of both cohorts were maintained on their original treatment arm and were followed for additional 18 months (up to month 30 after transplantation). At 24 months after transplantation, consenting patients underwent a per-protocol biopsy. The final goal of this extension phase of the study was to assess whether patients on SRL compared to those on CsA, in addition to express more Treg cells, were also more effectively protected from the late structural and

functional changes (as assessed by kidney biopsy evaluation and serial GFR measurements) characteristic of chronic allograft injury. All the investigators involved in patient care, evaluation of study outcomes - such as histology scores and kidney functional parameters -, and data handling and analyses were blinded to Treg counts.

As a further post hoc analysis, we stratified patients on the basis of Treg counts at 1 year: those with Treg counts above ($Treg^{+}$, n=10) or below ($Treg^{-}$, n=11) the median value. Again, we compared the outcomes in the two groups, in order to evaluate whether patients with higher Treg might exert a beneficial effect independently from the immunosuppressive therapy employed.



Schematic representation of study design.

Twenty-one primary kidney transplant Caucasian recipients were enrolled. Patients were randomly allocated on a 1:1 basis to either low-dose sirolimus (SRL group, n=11) or low-dose CsA (CsA group, n=10) added on to low-dose MMF and induction therapy with Campath-1H. Immunosuppressive treatments are indicated by arrows. At surgery, patients received 500 mg methylprednisolone, followed by a 30 mg infusion of Campath-1H. Methylprednisolone was also infused on day 1 (250 mg) and 2 (125 mg) post-transplant. Low-dose Sirolimus (Low SRL) was started on post-transplant day 1 (4 mg/day p.o., then adjusted to target trough levels of 5-10 ng/ml). Intravenous CsA (low CsA) was started soon after surgery (1-2 mg/kg/day); on day 1 post-transplant i.v. CsA was shifted to oral CsA (2 mg/kg/twice daily) and then adjusted to achieve trough blood concentrations of 120 to 220 ng/ml in the first month post-surgery, and of 70 to 120 ng/ml thereafter. In both groups, patients were also given MMF at the oral dose of 500 mg twice daily starting on day 1 postoperatively (target MPA concentration: 0.5-1.5 mg/ml).

Phenotypic and functional assessments are outlined on the left. Timing of each evaluation is marked with a cross.

Figure 1

Immunosuppressive protocol

Campath-1H (Alemtuzumab, Schering Plough, Milano, Italy) was given as a single intravenous infusion (30 mg, over 2 hours) intraoperatively on the day of transplant (day 0). Thirty minutes prior to the Campath-1H infusion, the patients were administered 500 mg methylprednisolone. Corticosteroids were also administered on day 1 (250 mg) and 2 (125 mg) post transplant. Thereafter, patients were free of steroids. Patients randomized to sirolimus (Rapamune, Wyeth, Rome, Italy) received the drug at the oral dose of 4 mg/day in a single morning administration starting on the day 1 after transplant. Sirolimus dosing was adjusted to target trough level of 5-10 ng/ml range. In the CsA-based group, the drug was started i.v. (at the dose of 1-2 mg/kg/day) just after surgery and shifted to the oral formulation (Neoral, Novartis Pharma, Basel, CH) on day 1 post transplant. CsA doses were adjusted to achieve trough blood concentration of 120 to 220 ng/ml in the first month post-surgery, and of 70 to 120 thereafter. Patients of both groups were also given MMF (Cell Cept, Roche, Milan, Italy) at the oral low dose of 250 to 750 mg twice a day starting on day 1 postoperatively according to total blood leukocyte count.

Postoperative monitoring

After kidney transplantation all patients were managed according to the standard protocol in use at our center. Serum creatinine levels, electrolytes, blood cell counts and other routine laboratory tests were monitored daily during hospital stay and up to 15 days after discharge. These parameters were then evaluated twice a week up to 1 month post transplant; then every one or two weeks up to 3 months post surgery and thereafter at monthly intervals. These evaluations were performed by the central laboratory of the

Azienda Ospedaliera OO. RR. Bergamo, Bergamo, Italy. Glomerular filtration rate (GFR) as an index of graft function, was estimated at monthly intervals by Walser formula [178]. Trough levels of mycophenolic acid were performed every week in the first month post-surgery, every 2 weeks during month 2-4, and every month thereafter. Trough morning blood sirolimus levels were monitored every 4-5 days in the first month post surgery, every 2 weeks during month 2-4, and every month thereafter by high-performance liquid chromatography (HPLC). Similarly, trough blood CsA levels were measured daily during hospitalization and then every **week** up to the first month post-transplant, every 2 weeks during month 2-4, and every month thereafter. Direct measurement of GFR and renal plasma flow (RPF) was performed by the plasma clearance of iohexol and of p-aminohippurate (PAH), respectively, every 6 months post-transplantation. At the same time intervals, the pharmacokinetic profile of SRL and CsA was also evaluated by high-performance liquid chromatography (HPLC). Trough levels of immunosuppressive drug and GFR and RPF were evaluated at the Clinical Research Center Villa Camozzi, Ranica (BG), Italy.

CMV antigenemia was monitored serially [179] and intravenous gancyclovir was administered when positive peripheral blood leukocyte count was > 20 cells/mm3, and continued for at least 1 week after the count had decreased to 0 cells/mm3. In CMV antibody negative recipients of graft from CMV antibody positive donors, intravenous gancyclovir was started on day 4 post-transplant regardless of CMV antigenemia and continued for 14 days. All patients were given standard anti-microbial prophylaxis, including trimethoprim-sulfamethoxazole or inhaled pentamidine (monthly) against *Pneumocystis carinii* for 6 months post-transplant.

At 2 years after transplantation, the two groups of patients underwent per-protocol graft biopsy unless medically contraindicated or if the patient refused consent. Rejection episodes were diagnosed on the basis of clinical judgement by the following criteria: 25% increase in serum creatinine concentration over the previous evaluation in the presence of expected drug blood/plasma trough levels, associated with renal ultrasound findings excluding urinary tract obstruction or other surgical complications. If clinical and/or laboratory signs indicated the occurrence of a rejection episode, renal biopsy was performed, unless medically contraindicated. If acute graft rejection was diagnosed, methylprednisolone i.v. pulses were administered. With positive response to treatment, the patients remained on the study, but oral steroid was resumed up to a maintenance daily dose of 8 mg. If graft rejection was steroid-resistant or a second acute rejection episode did occur, the patients were withdrawn from the study and treated with other more conventional immunosuppressive regimens.

Graft loss was determined as the time of re-establishment of long-term dialysis therapy or death. Delayed graft function was defined as the requirement of at least one dialysis session during the first 7 days after transplantation. All patients were followed after renal transplant for the incidence of acute rejection, graft loss, graft function, adverse events that required treatment or hospitalization, death, and drug blood levels.

Graft function measurement

Serum creatinine concentration was measured using a standard laboratory technique (Synchron CX9 ALX Pro, Beckman-Coulter, Milan, Italy).

Determination of glomerular filtration rate (GFR) and renal plasma flow (RPF) by plasma clearance of iohexol and para-aminohippuric acid

Plasma concentrations of iohexol and PAH were determined by high performance liquid chromatography (HPLC) as previously reported [180] with minor modification. Plasma samples were added 50 µl of 1,3-dimethyluric acid (200 µg/ml in phosphate buffer, pH 7.4) and deproteinized by adding 750 µl 5% perchloric acid and centrifuging. Twenty microliters of the supernatant was chromatographed using a System Karat HPLC equipped with variable wavelength detector (Beckman, Fullerton, CA, USA) and a 250 x 4 mm column packed with Lichrosorb C-18 (Merck, Darmstadt, Germany). Iohexol and PAH are eluted by a mixture of deionized water/acetonitrile (96:4 by volume, adjusted to pH 2.5 with phosphoric acid), pumped at a rate of 1.5 ml/min. Internal calibration curves of iohexol and PAH are prepared for each set of samples.

The iohexol plasma profile determined for each patient is analyzed by a one-compartment open model system. All data were fitted by a non-linear regression iterative pharmacokinetic program (data are weighted by $1/y^2$, where y is the observed value) on a personal computer. The clearance of iohexol was determined using the measurements from the timed period 120 minutes after the injection to the last sampling point, according to a one-compartment model (CL_1) by the formula:

$$CL_1 = Dose/AUC$$

(where AUC is the area under the plasma concentration-time curve) and then the value was corrected according to Bröchner-Mortensen [181], in order to estimate GFR (plasma clearance) by using the formula:

$$CL = 0.990778 \times CL_1 - 0.001218 \times CL_1^2.$$

GFR value was then normalized by the body surface area (GFR/ 1.73 m^2).

PAH clearance was calculated according to the formula:

$$RPF=Ro/C_{ss}PAH$$

where Ro is the infusion rate; $C_{ss}PAH$ is the PAH plasma concentration at the steady state (i.e. mean of PAH plasma concentrations measured at 150, 160, 170, 180 minutes from iohexol injection)

Renal plasma flow, estimated by plasma clearance of PAH, was then normalized by body surface area, and expressed as ml/min/1.73 m^2.

The plasma profiles were analyzed by one-compartment open model system, and calculated clearance of iohexol corrected according to the Bröchner-Mortensen formula [181]. With the same HPLC analytical run, the plasma concentration of PAH was also measured. GFR and RPF values were expressed per 1.73 m^2 of body surface area. Further details about the procedure are described in Appendix A.

Phenotypic and functional analyses of peripheral lymphocytes

Peripheral leukocyte count and lymphocyte phenotype

Peripheral blood cells were monitored serially by flow cytometry. The absolute count of each leukocyte population was determined using a single platform method. Twenty µl of MultiTEST four-color antibodies (BD Bioscience, San Jose, CA) and 50 µl of peripheral blood on K3EDTA were added to bead-containing TruCount tubes. The following MultiTest antibodies were used: CD3/CD4/CD8/CD45 and

CD19/CD16+56/CD3/CD45 (BD Bioscience) to determine the percentages and absolute counts of total T ($CD3^+$) $CD4^+$ ($CD3^+CD4^+$), $CD8^+$ ($CD3^+CD8^+$), natural killer (NK) ($CD3^-CD16^+CD56^+$), and B ($CD3^-CD19^+$) lymphocytes. Four hundred and fifty µl of FACS Lysing Solution was added and tubes were incubated for 20 minutes at room temperature. Samples were analysed using FACSCalibur cytometer and CELLQUEST software (BD Bioscience).

T lymphocyte subset immunophenotyping

For T lymphocyte subset immunophenotyping, frozen peripheral blood mononuclear cells (PBMC) obtained by Ficoll-Paque gradient centrifugation were used; PBMC were incubated with 20 µl of different fluorochrome conjugated murine monoclonal antibodies against human CD3, CD4, CD8, CD25, CD28, CD69, CD45RO antigens (BD Bioscience). In selected experiments labelling for CD3, CD4, CD25 and Ki-67 (BD Bioscience), a nuclear cell proliferation-associated antigen expressed in all active stages of the cells cycle, was performed. Thereafter, 200-300 µl of 1% paraformaldehyde was added to the cells, which were acquired immediately or stored at 4 °C in the dark for acquisition within 24 hours.

The labelling procedures were carried over as BD Bioscience technical data sheets. The samples were analyzed by four colour FACScan flow cytometer using the CELLQUEST Software (BD Bioscience). For each marker, blank samples with isotype matched control antibodies were analyzed.

FOXP3 expression

After gating for the $CD3^+$ cell population, $CD4^-$, $CD4^+CD25^-$, $CD4^+CD25^{low}$, $CD4^+CD25^{high}$, $CD8^+CD28^-$ T cells were isolated by cell sorting (FACSAria, BD). The purity of sorted cells was >99%. Total RNA was extracted from either PBMC or FACS sorted cells by PicoPure RNA isolation kit (Arcturus, Mountain View, CA, USA). Total RNA was reverse transcribed to cDNA using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was performed on a TaqMan ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with SYBR Green PCR Core reagents. PCR reactions were performed in triplicate, with 1 µl c-DNA and SYBR Green PCR Core reagents in a final volume of 25 µl. Primers were designed with Primer Express 2.0 Software (Applied Biosystems) and purchased from Sigma-Aldrich. To amplify human FOXP3 transcript the following primers were used, based on Genbank sequence AF277993: for (300nM) 5'-AGC CAT GGA AAC AGC ACA TTC -3'; rev (300nM) 5'- GAG CGT GGC GTA GGT GAA A-3'. Beta 2-microglobulin served as a housekeeping gene to assess the overall cDNA content. Beta 2-microglobulin primers were as follows: for (300nM) 5'-AAG TGG GAT CGA GAC ATG TAA GC -3'; rev (300nM) 5'-TCA TCC AAT CCA AAT GCG G - 3'. After an initial holding step of 2 minutes at 50°C and 10 minutes at 95°C, samples were cycled 40 times at 95°C for 15 seconds and 60°C for 60 seconds. Melting curve analysis showed a single dissociation pick either for FOXP3 or beta 2-microglobulin PCR products, confirming the specificity of the reactions. No amplification was found in control reactions without c-DNA.

Similar amplification efficiencies were demonstrated for both the target and the housekeeping gene by analyzing serial cDNA dilutions, showing an absolute value of the slope of log input cDNA amount versus Δ threshold cycle (Ct) (Ct target- Ct

housekeeping gene) of <0.1. Then the ΔΔCt equation was used to compare the FOXP3 gene expression in each sample with the expression in $CD3^+CD4^+$ cells from a pool of healthy subjects taken as reference (calibrator). Results were expressed as arbitrary units (AU) taking the expression in the calibrator as 1.

Measurement of lymphocyte alloreactivity

Peripheral blood mononuclear cell (PBMC) and spleen cell sampling

Either recipient or living donor PBMCs were isolated from heparin-treated blood. The blood was diluted with an equal volume of balanced salt solution and layered carefully over Ficoll-Paque PLUS (Pharmacia, Uppsala, Sweden) in a centrifuge tube. After centrifugation at room temperature (2400 rpm for 20 min, without brake), the following layers will be visible in the column, from top to bottom: plasma and other constituents, PBMC, Ficoll-Paque, and erythrocytes and granulocytes which should be present in pellet form. This separation allows easy harvest of PBMC using a sterile transfer pipet. The cells were then washed with Phosphate Buffer Saline solution (PBS) to remove the platelets and centrifuged (1800 rpm for 8 min). PBMC were thereafter resuspended in complete RPMI supplemented with L-glutamine and penicillin/streptomycin and 20 % heat inactivated pooled human serum type AB (Sigma, St Lous, MO). The cells were counted using a hemocytometer, then aliquoted and frozen in the presence of 10% DMSO. Subsequently, PBMC were placed in liquid nitrogen vapor.

Deceased donor spleens were cut into small fragments and filtered through a stainless steel screen to obtain a total spleen cell suspension. Cell suspension was then filtered through a 40 μm cell filter. For erythrocyte lysis, cells were treated with ACK (0.15 M NH_4Cl, 10mM $KHCO_3$, 0.1 mM EDTA, pH 7.4) on ice for 8 minutes, then washed whit

PBS. Splenocytes were thereafter resuspended in complete RPMI supplemented with L-glutamine and penicillin/streptomycin and 20 % heat inactivated pooled human serum type AB. The cells were counted using hemocytometer, then aliquoted and frozen in the presence of 10% DMSO. Subsequently, splenocytes were placed in liquid nitrogen vapor.

Before using in MLR and ELISPOT experiments, spleen cells were depleted of $CD2^+$ cells by immunomagnetic cell isolation using Dynabeads CD2 kit (Dynal Biotech, Oslo, Norway).

Cryopreservation of PBMC and splenocytes

Freshly isolated PBMC and splenocytes were resuspended at 1 x 10^7 viable cells/ml in RPMI + 20% serum AB. In each 1.5 ml cryovial were placed 750 µl of this suspension and then were added drop by drop other 750 µl of a solution with RPMI + 20% serum AB + 20% DMSO.

Cryovials were placed in a Mr. Frosty-style freezing container that has been filled with 100% isopropanol according to the manufacturer's instructions. The freezing container was thereafter put at –80°C overnight and then cryovials were transferred into liquid nitrogen vapor.

Thawing of PBMC and splenocytes

Cryovials were transferred from liquid nitrogen vapor to a 37°C water bath. Then, suspension was diluted into warm media and centrifuged at 1800 rpm for 8 minutes. The supernatant was decanted, and the tube was gently flicked with a finger to break up the pellet. Then, cell were resuspended in 3-5 ml of RPMI + 20% human serum. Cell

number and viability by Trypan blue staining were assessed thereafter.

Mixed lymphocyte reaction (MLR)

For MLR, $1x10^5$ recipient PBMC (in 100 μL) were added in triplicate wells in a round-bottomed 96-well plate. Irradiated (4000 RAD) stimulator cells (100,000) isolated from the donors (either PBMC from living donors or CD2-depleted spleen cells from cadaveric donors), from third-party subjects (either PBMC or CD2-depleted spleen cells) or from the recipient (PBMC, self control combination) were added to the wells. Third-party controls were chosen, to the extent possible, so that the number of mismatches for HLA was the same as that between the donor and recipient. Aliquots of responder PBMC were also incubated with medium alone (negative controls). The plates were then incubated for 6 days at 37°C, 5% CO_2 and were pulsed with 1 μCi [^{3}H] thymidine during the last 16 h. Thereafter, cells were harvested using an automated harvester. [^{3}H] thymidine incorporation by T cells was used as a parameter of cell proliferation and measured by a beta-counter. The mean counts per minute (cpm) were determined and the stimulation index (SI) was calculated by the ratio of the cpm obtained in the presence of allogeneic combinations (donor or third party) to the cpm in the control wells (self combination).

Enzyme-linked immunosorbent spot (ELISPOT) assays

ELISPOT assays were performed using BD ELISPOT Human IFNγ reagents. Responder PBMCs from the recipient were placed in 96-well ELISPOT plates (Millipore, Billerica, MA) pre-coated with capture anti-IFN-γ at the concentration of 300,000 per well. Irradiated (4000 RAD) stimulator cells (300,000) from the donors

(either PBMC from living donors or CD2-depleted spleen cells from cadaver donors), from third-party subjects (either PBMC or CD2-depleted spleen cells) or from the recipient (PBMC, self control combination) were added to the wells and the plates were incubated overnight at 37°C, 5% CO_2. Aliquots of responder PBMC were also incubated with medium alone (negative controls) or in the presence of 10 μg/mL phytohemagglutinin (PHA, positive controls). Each combination was run in triplicate wells. The assays were then carried out according to the manufacturer's instructions. The resulting spots were counted on a computer-assisted Immunospot image analyzer (Aelvis Elispot Scanner system). Results are the mean value of IFN-γ spots/300,000 recipient PBMC stimulated with donor or third party cells after subtracting IFN- γ spots in negative controls (usually 2 or less).

Renal transplant per-protocol biopsy

Renal biopsy procedure

Patients were admitted to our Transplant Center the day before the procedure. Those on antiplatelet or anticoagulant therapy were asked to interrupt this therapy one week before the day planned for the procedure. Coagulation profiles and platelet counts were evaluated at the admittance and those patients showing abnormalities did not **undergo** the biopsy. Also patients with bleeding time higher than 10 minutes did not underwent the procedure. All patients were informed about the potential risks of the procedure and signed an informed consent approved by the Ethical **Committee** of our Hospital.

The biopsies were all performed by the staff radiologists with automated biopsy gun under sonographic guide. The Biopty gun, a sterilized spring-loaded instrument, was fitted with an 18-gauge needle, and the tip of the needle was placed just inside the renal

capsule of the lower pole of the transplanted kidney. Real-time sonographic guidance was used to determine placement. The needle excursion of 2.3 cm after firing was taken into account during needle positioning. After the biopsy, the tissue cylinder was inspected under the reflected light microscope. The biopsy was repeated if this check failed to reveal glomeruli in the tissue cylinder. Generally, a single biopsy was enough.

Kidney biopsy processing

The kidney samples of patients were left in Dubosq-Brazil fixative for four hours. After the fixation step, the samples were dehydrated in growing concentrations of ethanol (50, 70, 90, 100 % for five minutes each). Then, after one hour in toluene, the samples were collected into the small stainless steel base molds (Electron Microscopy Sciences, Rome, Italy), completely immersed into the paraffin and left for two hours at 60 °C. At this temperature the paraffin is in the liquid state and it can infiltrate the tissue. After the two hours, the samples were embedded in paraffin using special plastic rings and left at room temperature to permit the solidification of the paraffin. Finally, the kidney samples included into the blocks of paraffin were cut by microtome (LKB, Bromma, Historange Microtome) or stored at room temperature.

Staining protocol

Dubosq-Brazil fixed, paraffin embedded kidney sections (3 μm) were deparaffinized, rehydrated and incubated for 30 minutes with 0.3% H_2O_2 in methanol to quench endogenous peroxidase. Tissues were treated with proteinase-K (20 μg/ml, Sigma-Aldrich, Milan, Italy) for 10 minutes at 37°C, followed by microwave (twice for 5 min in citrate buffer 10 mM, pH 6 at operating frequency of 2450 MHz and 600-W power

output) and citrate buffer (15 min) incubations for antigen retrieval. A polyclonal antibody against human C4d (C4dpAb, Biomedica, Vienna, Austria) was diluted 1:50 and added overnight at 4°C. Subsequent steps included incubations with the secondary antibody (biotinylated goat anti-rabbit IgG, Vector Laboratories), avidin-biotin peroxidase complex (ABC) solution, and finally the development with diaminobenzidine. The sections were then counterstained with Harris hematoxylin (Biooptica, Milan, Italy). A set of biopsies comprising samples from each patient group was processed and developed simultaneously. Negative controls were obtained by omitting the primary antibody on a second section present on all the slides.

Histology injury scoring

Chronic allograft injury was diagnosed where diffuse tubular atrophy or diffuse interstitial fibrosis were documented alone or in association with interstitial inflammation, arteriosclerotic lesions, or glomerulopathy [34, 182, 183]. Semiquantitative analysis of changes was performed according to chronic allograft damage index (CADI) [184], by an investigator who was blinded to the clinical status of the patients. The CADI score is the sum score of six histology parameters, including (a) interstitial inflammation and (b) fibrosis, (c) tubular atrophy, (d) mesangial matrix increase and (e) sclerosis of the glomeruli, and (f) intimal proliferation of the blood vessels. Each individual parameter was scored from 0 to 3. Evaluation and scoring of C4d expression was also done. The signal intensity at glomerular and tubulo-interstitial level was graded on a scale of 0 to 3 (0, no staining; 1, weak staining; 2, staining of moderate intensity; 3, strong staining).

For quantification of histology changes in each biopsy, at least three stained slides were used: one with hematoxylin-eosin, one with Masson's trichrome, and one with periodic acid-Schiff staining.

Monitoring of immunosuppressive drug levels

Mycophenolate mofetil

Trough levels of mycophenolic acid were assayed every week in the first month post-surgery, every 2 weeks during month 2-4, and every month thereafter.

Total and free MPA plasma concentrations were measured by HPLC, introducing some modifications to already published methods [185]. MPAG was estimated as MPA after hydrolysis mediated by β-glucuronidase.

Sirolimus

Sirolimus levels were monitored every 4-5 days in the first month post surgery, every 2 weeks during month 2-4, and every month thereafter by high-performance liquid chromatography (HPLC). For the preparation of in-house QCs and calibration standards, different pools of whole blood samples from 15 healthy volunteers was used. In addition, subsequent to signing of an informed consent form, EDTA anticoagulated whole blood samples were obtained from 30 kidney, heart and liver transplant recipients not given SRL.

Standard samples of SRL (purity ranging from 97 to 98%, according to different batches) and 32-O-desmethoxyrapamycin (internal standard, IS) were generous gifts from Wyeth-Ayerst Research Laboratories (Princeton, NJ) and furnished with adequate information on drug source, lot number, expiration date and certificate of analysis.

Cyclosporine

Trough blood CsA levels were measured daily during hospitalization and then every weeks up to the first month post-transplant, every 2 weeks during month 2-4, and every month thereafter. CsA was measured by HPLC with UV detection, using the method from Kahn et al [186], with some modifications, as described in the "Solution and Instruments" section.

Sample size and statistical analyses

Sample size

This is mainly an immunological, clinical research project, so no assumption was made on the power of our present study to detect differences in PBMC phenotypic and functional characteristics and in clinical outcome variables between the two groups of transplant patients who received, after Campath-1H induction, maintenance therapy with either low-dose SRL or CsA both combined with low-dose MMF. The rationale for this approach was that this was a pilot, explorative study and that outcome data might have provided the background for designing future adequately powered trials to definitely assess the role of different immunosuppressive strategies on the number and *in vitro* function of Treg and the impact of these cells on clinical outcomes of kidney transplant patients.

Statistical analyses

Baseline characteristics of patients were compared by χ^2test or by t test as appropriate. SRL and CsA groups were compared for factors that might affect the outcome, including recipient gender, age at transplant, cold ischemia time, DGF and the degree of

Human Leukocyte Antigen (HLA) mismatch. Biopsy data were analyzed by the nonparametric Kruskal-Wallis test for multiple comparisons.

Clinical and biochemical parameters at different time points post transplantation were compared by means of analysis of covariance (ANCOVA) including the corresponding value at 6 months in the model. Patient and graft survival were compared by the log rank test. The GFR and RPF slopes were calculated on the basis of values measured at 6, 12, and 18 months after transplant. The statistical significance level was defined as $p < 0.05$. Calculations were performed using SAS v.9 and MedCalc (Gent, Belgium) software.

SOLUTIONS AND INSTRUMENTS

PBMC isolation and storage

Ficoll

Ficoll-Paque™ PLUS is a sterile, ready to use density gradient medium for purifying lymphocytes in high yield and purity from small or large volumes of human peripheral blood, using a simple and rapid centrifugation procedure.

Ficoll-Paque PLUS is an aqueous solution of density 1.077 + 0.001 g/ml containing 5.7 g Ficoll 400 and 9 g sodium diatrizoate with 0.0231 g calcium disodium ethylenediamintetraacetic acid in every 100 ml. Ficoll 400 is a synthetic high molecular weight (Mw 400 000) polymer of sucrose and epichlorohydrin which is readily soluble in water. The molecules of Ficoll 400 are highly branched, approximately spherical and compactly coiled with a Stokes' radius of a about 10 nm. Ficoll 400 has a low intrinsic viscosity (17 ml/g) compared with linear polysaccharides of the same molecular weight (cf. dextran Mw 400 000: /h/ 49 ml/g) and solutions of Ficoll 400 have low osmotic pressures.

Phosphate buffered saline (PBS)

Phosphate buffer saline (PBS) is a buffer solution containing sodium chloride, sodium phosphate and potassium phosphate that helps to maintain a constant pH. It is isotonic and non-toxic to cells. We started from a 10X Concentrate without Ca and Mg (GIBCO) that, when diluted to a 1X concentration, yielded a phosphate buffered saline solution with a phosphate buffer concentration of 0.01 M and a sodium chloride concentration of 0.154 M. The solution pH was 7.4.

RPMI medium

RPMI medium with L-glutamine (Sigma-Aldricht) plus 20% human serum was used as cell medium.

Human serum

For PBMC and splenocyte cultures, human serum (Sigma Aldricht) from a pool of AB males was used instead of fetal calf serum to minimize the potential ractivity of the cells. Serum was obtained by a pool of healthy donors. Each donor was tested for and found non-reactive for Hepatitis B& C and non-reactive for Human Immunodeficiency Virus (HIV) antibody by ELISA. Serum was added at a 20% concentration into RPMI solution. Before use, the complement serum was inactivated by heating for 56°C for 30 minutes.

Dynabeads CD2 kit

The Dynabeads CD2 kit (Dynal Biotech, Oslo, Norway) is intended for magnetic isolation or depletion of $CD2^+$ cells. Dynabeads are mixed with the sample in a tube. The Dynabeads will bind to the target cells during a short incubation, and then the bead-bound cells are separated by a magnet. Dynabeads CD2 are provided in phosphate buffer saline (PBS), pH 7.4, containing 0.1% bovine serum (BSA) and 0.02% sodium azide. Dynabead concentration is 4×10^8/ml.

Dimethyl sulfoxide (DMSO)

DMSO is a solvent used as a cryoprotectant. Added to cell media, it prevents the cells dying as they are frozen. Cells were suspended in standard media plus serum and 10% DMSO, put in a Freezing container to -80°C and then, 24 hours later, placed into liquid nitrogen vapor.

5100 Cryo 1°C Freezing Container, "Mr. Frosty"

This device provides the critical, repeatable -1°C/minute cooling rate required for successful cell cryopreservation and recovery. It requires 100% isopropyl alcohol and mechanical freezer. Cells were frozen with this freezing container to -80°C and then were placed into liquid nitrogen vapor.

PBMC phenotyping

TruCount Tubes

Each TruCOUNTTube contains a lyophilized pellet that dissolves during sample preparation, releasing a known number of counts of lymphocyte subsets. By gating the bead population during analysis, it is possible to calculate subset absolute counts.

FACS Lysing Solution

FACS Lysing Solution (Becton Dickinson), 10X concentrate, is provided as 100 mL of a proprietary buffered solution containing <15% formaldehyde and <50% diethylene glycol. Before use, the solution is diluted 1:10 with deionized water. The prepared solution is stable for 1 month when stored at room temperature.

Monoclonal antibodies

Murine monoclonal antibodies against human antigens were used. For leukocyte subset counting the Multitest antibodies (BD Bioscience) CD3/CD4/CD8/CD45 and CD19/CD16+56/CD3/CD45 were used. The following florochromes were used for different T cell subset antigens: CD3 PerCP, CD4 FITC (or APC-Cy7, according to the different combinations of the other antibodies), CD8 PE (or APC-Cy7), CD25 PE, CD28PE, CD69 FITC, CD45RO PE, Ki-67 FITC (used after cell permeabilization). Isotype mouse $IgG_{1,k}$ (FITC, APC-Cy7, PerCP, or PE) and IgG_{2a}PE were used as controls.

All the antibodies were purchased from BD Bioscience.

BD FACSAria

The BD FACSAria flow cytometer is an automated multicolor flow cytometry system that performs both cell phenotype analysis and sorting.

BD CellQuest software

BD CellQuest software allows to acquire and analyze data from flow cytometer on a Macintosh® computer. Working in the CellQuest Experiment window, it is possible to create several types of plots, including multicolor contour plots and overlaid histograms, and generate statistics for dot plots, histograms, density plots, and contour plots. We used this software both for analyzing data on the phenotypic profile of PBMC and to sort them.

FOXP3 expression

PicoPure RNA isolation kit

The PicoPure RNA Isolation Kit (Arcturus, Mountain View, CA, USA) was developed to efficiently isolate total RNA from a small number of cells. The isolation protocol consists of extracting cellular RNA, then loading the extract onto the MiraCol™ Purification Column to bind the RNA. After washing away impurities, the RNA elutes in only 10 µl of buffer, ready for use.

Total Reagents and Supplies in kit:

- Conditioning Buffer
- Extraction Buffer
- 70% Ethanol
- Wash Buffer
- RNA purification columns with collection tubes
- Microcentrifuge tubes

Superscript II Reverse Transcriptase

Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) is an engineered version of M-MLV RT with reduced RNase H activity and increased thermal stability. The enzyme is purified to near homogeneity from *E. coli* containing the modified pol gene of Moloney Murine Leukemia Virus. The enzyme can be used to synthesize first-strand cDNA at higher temperatures than conventional M-MLV RT, providing increased specificity, higher yields of cDNA, and more full-length product. It can generate cDNA up to 12.3 kb.

Components of the kit:

- SuperScript™ II RT
- 5X First-Strand Buffer (250 mM Tris-HCl, pH 8.3 at room temperature, 375 mM KCl; 15 mM $MgCl_2$)
- 0.1 M DTT

SYBR® Green PCR Core Reagents kit

Includes AmpliTaq Gold® DNA Polymerase, AmpErase® UNG, dNTP Mix with dUTP, SYBR® Green PCR Buffer and mM MgCl2 Solution to perform real time PCR analysis. Direct detection of PCR product is monitored by measuring the increase in fluorescence caused by the binding of SYBR Green dye to double-stranded (ds) DNA.

TaqMan ABI Prism 5700 Sequence Detection System

TaqMan ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) is a real-time PCR machine which enables detection and quantification of PCR products in real-time using either SYBR green reagents or Taqman probes. It is possible to monitor PCR reactions cycle by cycle enabling quantification and rapid analysis of many different targets.

PBMC functional assays

MLR

Tritiated thymidine

One μCi titriated thymidine (Amersham) was added to the MLR cultures 16 h before harvesting. It was used under the standard safety rules for work with radioactive compounds.

ELISPOT

ELISPOT kit

The enzyme-linked immunospot (ELISPOT) assay is a powerful tool for detecting and enumerating individual cells that secrete a particular protein *in vitro*. Based on the sandwich enzyme-linked immunosorbent assay (ELISA), the ELISPOT assay derives its specificity and sensitivity by employing high affinity capture and detection antibodies and enzyme-amplification. The sensitivity of the assay lends itself to measurement of even very low frequencies of analyte-producing cells (eg, 1/300,000).

The ELISPOT kit (BD) included:

- 2 Pre-coated ELISPOT plates
- Biotinylated Detection Antibody
- Enzyme Conjugate (Streptavidin-HRP)
- Assay Diluent
- Wash Concentrate (20×)
- PBS (10×)
- AECSubstrate Buffer

- AECChromogen

Aelvis Elispot Scanner system

The A.EL.VIS (Automated ELispot Video-analysis System) ELISPOT analyser is an automated instrument for the analysis of ELSPOT data. When scanning a 96 well membrane or foil the plate image is transferred to the connected PC using intelligent image analysis algorithms to separate the single wells. The extracted well images are individually stored and subsequently analysed by EliAnalyse software. The software is theoretically divided in two parts. Firstly the acquisition mode enables definition of data storage, secondly the analysis mode.

Processing of biopsy samples

Fixative for light microscopy

The fixative used for light microscopy, Dubosq-Brazil, was prepared adding 150 ml of ethanol at 80 % (diluted in water) to 60 ml of formaldehyde at 40 % (commercially available at this percentage by Carlo Erba Reagenti) and to 15 ml of acetic acid glacial (Carlo Erba Reagenti). Finally, 1 g of picric acid (Carlo Erba Reagenti, Milan, Italy) was added to the solution.

Solutions for Hematoxylin-eosin staining

Haematoxylin:

- Haematoxylin 6.0 g
- Alluminium Sulphate 4.2 g
- Citric Acid 1.4 g

- Sodium Iodate 0.6 g
- Ethylene Glycol 269 ml
- Distilled Water 680ml

Eosin:

- Eosin Yellowish 1.0 g
- Distilled Water 100 ml

Scott's tap water:

- In a beaker containing 1L distilled water, 20g sodium bicarbonate and 3.5g magnesium sulphate were added and mixed thoroughly to dissolve the salts. Using a filter funnel, the solution was transferred into a labelled bottle.

Solutions for Masson's trichrome staining

Bouin's Solution:

- Picric Acid, saturated aqueous solution 75.0 ml
- Formaldehyde, 37-40% 25.0 ml
- Glacial acetic acid 5.0 ml

Weigert's Iron Hematoxylin:

Solution A

- Hematoxylin 10.0 g
- Alcohol, 95% 1,000.0 ml

Solution B

- Ferric chloride, 29% aqueous solution 20.0 ml
- Distilled water 475.0 ml
- Glacial acetic acid 5.0 ml

Working Solution

- A mix of equal parts of solutions A and B

Biebrich Scarlet-Acid Fuchsin Solution

- Biebrich scarlet, 1% aqueous solution 360.0 ml
- Acid fuchsin, 1% aqueous solution 40.0 ml
- Glacial acetic acid 4.0 ml

Phosphomolybdic-Phosphotungstic Acid Solution

- Phosphomolybdic acid 25.0 g
- Phosphotungstic acid 25.0 g
- Distilled water 2,000.0 ml

Aniline Blue Solution

- Aniline Blue 25.0 g
- Glacial acetic acid 20.0 ml

1% Acetic Acid Solution

- Glacial acetic acid 1.0 ml
- Distilled water 99.0 ml

Solutions for periodic acid-Schiff staining

0.5% Periodic Acid Solution:

- Periodic acid 0.5 g
- Distilled water 100 ml

Schiff Reagent

Mayer's Hematoxylin Solution

Monitoring of immunosuppressive drug levels

Mycophenolic acid (MPA)

Human plasma, chemicals and materials

Calibration standards and QCs were prepared using pools of plasma samples from 15 healthy volunteers and from 20 kidney and liver transplant recipients not given MMF or EC-MPS (used to test potential concomitant medications).

Standards of MPA and MPAG were initially donated by Roche Pharmaceuticals (Palo Alto, CA). After 2002, MPA was bought from Sigma (St Louis, MO), together with p-toluic acid (PTA, used as internal standard). All the batches of MPA have a purity > 98% and were provided with the certificate of analysis. Acetonitrile, methanol were HPLC grade and were purchased by BDH (UK), all other chemicals were from Sigma. HPLC quality deionized water was prepared using Milli Q50 (Millipore, Bedford, MA). Bond-Elut C18, 200 mg, 3 ml cartridges were obtained from Varian (Leinì, Italy).

Stock solutions, calibrators, and quality control standards

Stock solutions, containing 10, 100 mg/L of MPA and 50 mg/L PTA were prepared in methanol and stored at 4°C until use. Aliquots of the stock MPA solutions were diluted with drug free plasma to give 6 calibrators (0.1, 1, 5, 10, 20 and 40 MPA mg/L). Two in-house QCs were prepared in drug-free plasma with a final concentration of 2 and 20 mg/L MPA. Calibrators and QCs were stored at -20°C until use.

Sample preparation

Over 95% of MPA is bound to albumin, whereas only a limited amount of the drug is distributed within blood cells [187]. Therefore, plasma is the matrix of choice for the assessment of MPA levels in the blood.

Five hundred microliters of plasma was mixed with 1.5 mL of water, 50 μL of internal standard and 750 μL of 0.1 N HCl. The mixture was applied to a C18 solid phase extraction column pre-conditioned with 2 mL of methanol followed by 2 mL of water. The column was dried and then eluted with 1 mL of methanol/0.1 N acetate buffer (80:20 v/v) pH 4. Samples were collected in HPLC vials.

HPLC apparatus and conditions

A System Gold HPLC equipped with a model 166 UV detector set at 254 nm and a model 507 autosampler (Beckman, Fullerton, CA) were used. The autosampler was kept at room temperature, and a 50 μL aliquot sample was injected. The separation was carried out at room temperature using a C18 column, 250 x 4.6 mm, 5 μm (Hypersil BDS, Hewelett Packard, Ge). A guard column (LiChrosper 100 RP-18, 5 μm) was placed just before the column. The mobile phase for elution of the column was 45% acetonitrile and 55% aqueous phosphoric acid (0.05%), at flow rate of 0.8 mL/min. Data were collected and processed using a 32 Karact software for HPLC system (Beckman, Fullerton, CA).

Assay validation

Method performance was determined in accordance with the FDA Guidance for Bioanalytical Methods Validation for Human Studies.

Sirolimus

Stock solutions, calibrators and quality control standards

Stock solutions containing 50 and 100 μg/mL were appropriately prepared in methanol for SRL and IS, respectively. SRL working solutions of 100, 500, and 2000 ng/mL were prepared in 50/50 methanol/water, and for IS a working solution of 1000 ng/mL was prepared in methanol. All the solutions were stored at -20 °C.

Taking into account the therapeutic range of SRL trough levels, calibrator samples were prepared mixing appropriate volumes of SRL from stock working solutions to EDTA anticoagulated human whole blood from healthy volunteers to achieve different concentrations from 2.5 to 60 ng/mL (2.5, 5, 10, 15, 20, 40, and 60 ng/mL). Calibrators were prepared by diluting each spiking solution to 10 ml with K_3EDTA control human whole blood in 10 ml volumetric flasks. The flasks were stopper and shaken to mix. Pools are measured into 1 mL aliquots in polypropylene tubes and frozen at –20°C until use. Three in-house QCs, representing the low, medium and high concentrations, were prepared in drug-free whole blood with a final concentration of 3, 10 and 30 ng/mL SRL. Calibration, QCs and reference standards were aliquoted and stored at -20°C until use.

Sample preparation

SRL is extensively distributed in red blood cells, independently of concentration and temperature [188], so we decided to use whole blood as the preferred matrix for method validation.

One millilitre volume of whole blood sample was pipetted into disposable polypropylene tubes and supplemented with 50 μL of IS solution (1000 ng/mL). The tubes were vortex-mixed for 40 seconds; 1.5 mL of zinc sulphate solution was first added followed by a 1.5 mL acetone. The tubes were vortex-mixed for a further 50-60 seconds and centrifuged at 3000 g for 5 minutes at room temperature. The clear supernatant was poured into another polypropylene tube, diluted with 2 mL distilled water, mixed and loaded onto a Bond-Elut cartridge (preconditioned with 1 mL acetonitrile followed by 1 mL methanol and finally by 1 mL distilled water) placed on a Vac Elut 20 Manifold (Varian). The Bond-Elut cartridges were washed with 1.5 mL of 70% methanol/30% water. In each step the solvent was allowed to drop out from the cartridge. Then 500 μL hexane was added and the column was allowed to go dry under vacuum. SRL and IS were eluted in polypropylene tubes with 1 mL acetonitrile. In all steps the flow rate did not exceed 1 mL/min. The eluate was taken to dryness either under a gentle nitrogen stream in a water bath at 37 °C or in a model RC 10.09 centrifugal evaporator (Jouan, Saint-Herblain, France) and the residue was dissolved in 0.15 mL of water-methanol-acetonitrile (40/30/30) and transferred in a polypropylene vial. Internal calibration curves for SRL were prepared for each set of samples. At least 60 samples (including controls and calibration curve) can be extracted in 4 h and processed by HPLC in less than 20 h.

HPLC apparatus and conditions

A System Gold HPLC equipped with a model 166 UV detector set at 278 nm and a model 508 autosampler (Beckman, Fullerton, CA) with the sample tray kept at 4 °C, were used. A 90 μL aliquot of sample was injected onto reversed-phase C18, 5 μm,

guard column (Alltima, 7.5 x 4.6 mm, Alltech, Sedriano, Milan, Italy) connected to a 75 x 4.6 mm column packed with Ultrasphere C8, 3 μm (Beckman) heated at 50 °C by a Model 880 oven (Spark-Holland, Emmen, The Netherlands) and was eluted by a mixture of distilled water/methanol/acetonitrile (34/30/36) pumped at a rate of 1 ml/min. Due to the high percentage of the organic phase that may dry off, resulting in increased retention time, the mobile phase was prepared every one or two days before analysis, filtered and degassed under vacuum using a polycarbonate 0.4-μm membrane. An in-line filter (0.5 μm) was placed between the autosampler and the column. Data were collected and processed using a 32 Karat software (Beckman, Fullerton, CA).

Method Validation

Method performance was determined in accordance with the FDA Guidance for Bioanalytical Methods Validation for Human Studies, as described above.

For the Proficiency Test initially 78 blinded samples, packaged as 5 batches of samples each, were analyzed. In addition, ongoing proficiency was tested by analyzing 3 blinded samples from the Reference Laboratory every month.

Cyclosporine

Human whole blood, chemicals and materials

Calibration standards, QCs and blanks were prepared using pools of whole blood samples from healthy volunteers (n=15) and from kidney (n=10) and liver (n=10) transplant recipients not given CsA.

CsA and cyclosporine D (IS) were kindly supplied by Novartis Pharma (Basel, CH), with a declared purity of 100%. Methanol and acetonitrile (BDH, UK) were of HPLC

grade. All other solvents were of analytical grade (Sigma, St Louis, MO). Deionized water was prepared using a Milli Q50 system (Millipore, Bedford, MA).

Stock solutions, calibrators and QCs

Stock solutions containing 100 mg/L of CsA and IS, as well as working solutions (10 mg/L for CsA and 20 mg/L for IS) were prepared in methanol. For calibration of the analytical system appropriate volumes of CsA from stocked working solutions were added to 1 mL EDTA anticoagulated human whole blood to achieve 7 different CsA concentrations (20, 50, 100, 200, 500, 1000 and 2000 ng/mL). QCs were prepared spiking known volumes of CsA from working solutions to drug free whole blood in order to obtain three concentrations (30, 300, 900 ng/mL CsA). CsA solutions, calibrators and QCs were stored at -20°C.

Sample preparation

To one mL of peripheral vein blood samples we added IS (50 μL), hydrochloric acid 0.2N (1 mL), and heptane (1 mL). The mixture was vortexed for 10 sec to lyse the blood cells. Subsequently we added diethyl ether (8 mL) and each tube was tightly capped. Extraction of CsA was effected on a reciprocal shaker. The organic phase was clarified by centrifugation for 15 min at 3000 RPM. The ether layer was decanted into a clear glass tube and washed with sodium hydroxide 0.1 N (1mL). Following a second centrifugation for 10 min, the ether layer was transferred into a clean glass tube and evaporated to dryness under a gentle nitrogen stream in a water bath at 37°C. The residue was redissolved in 200 μL of the mobile phase and washed by vortexing for 30

sec with heptane (1 mL). The sample was finally centrifuged (10 min at 3000 RPM) and the lower aqueous layer transferred in a polypropylene vial.

HPLC apparatus and conditions

A system Gold HPLC with a UV detector set at 214 nm and a model 580 autosampler (Beckman, Fullerton, CA) was used. A 50 μL of aqueous layer was injected into a C-8 HPLC column (150 x 4.6 mm, 5 μm, Beckman) heated at 72°C by a LC oven 101 (Perkin Helmer, Milan). Isocratic liquid chromatography separation was carried out using a mobile phase of water/methanol/acetonitrile (27/32/41) at a flow rate of 1 mL/min. Data were collected and processed using a 32 Karact software (Beckman).

Method Validation

Method performance was determined in accordance with the FDA Guidance for Bioanalytical Methods Validation for Human Studies, as described above.

Since July 2003, this method is enrolled in the Cyclosporine international Proficiency Testing Scheme. Ongoing proficiency is tested by analyzing 3 blinded samples from the Reference Laboratory every month.

RESULTS (I Part)

1. Phenotypic and functional profiles of peripheral blood mononuclear cells (PBMCs) in kidney transplant patients who received Campath-1H induction and low-dose SRL or low-dose CsA, both in addition to low-dose MMF in a steroid-free regimen.

Introduction

The best maintenance therapy to combine with Campath-1H induction is unknown and the immune regulatory mechanisms that allow prevention of acute rejection with lower than conventional doses of maintenance immunosuppression after Campath-1H induction are still unclear. Notably, different maintenance immunosuppressive regimens might differently affect phenotype and function of peripheral lymphocytes.

Thus, in the first part of the study, we aimed to define the phenotypic and functional profile of peripheral lymphocytes from kidney transplant patients given Campath-1H induction and low doses of SRL or CsA, both combined with low doses of mycophenolate mofetil as maintenance immunosuppression.

Specific aims

Specific aims of the first part of the project were:

i) To assess the depleting effect of a single 30 mg dose of campath-1H on peripheral leukocytes in kidney transplant recipients;

ii) to study the impact of SRL and CsA, each combined with MMF on the recovery of different leukocyte subsets in kidney transplant patients after Campath-1H induction;

iii) to evaluate whether the two immunosuppressive regimens differently affected Treg number and function after Campath-1H induction;

iv) to measure T cells alloreactivity in kidney transplant patients who received Campath-1H induction and maintenance immunosuppression with low-dose MMF and low-dose SRL or CsA;

v) to evaluate whether potentially reduced alloreactivity against donor antigens depends on the presence of Treg or on anergy.

Results

Demographic and baseline clinical characteristics of patients included in the study

Table 1 shows donors' and recipients' baseline characteristics, cold and warm ischemia time, and HLA -A, B and DR mismatches of patients included in the study and randomized to maintenance therapy with SRL or CsA therapy. All patients received kidney transplant from deceased donors, except for two of those on CsA whose donors were living-related. Donors' age, weight, and gender distribution were very similar between the two groups. Cold ischemia time for grafts from deceased donors ranged from 14 to 18 hours, whereas warm ischemia time was around 30 minutes in both groups. Recipients' age, weight, and gender distribution were similar as well between the two groups and between donors and recipients of the same randomization arm. The number of donor-recipient HLA mismatches was virtually identical between SRL (4.0±1.4) and CsA patients (4.0±1.2), ranging from 1 to 5. Major causes of renal failure

were IgA nephropathy (n = 6), pyelonephritis (n = 3), and polycystic kidney disease (n = 3). For 6 patients the diagnosis of end-stage renal disease was unknown. None of the patient in either group was diabetic (Table 1).

Effect of Campath-1H induction on peripheral leukocytes: extent of depletion and time-course of recovery in the two groups of kidney transplant recipients

Depletion of circulating leukocytes after Campath-1H induction

We first studied the effect of Campath-1H depletion on leukocyte subset counts and phenotype in the two cohorts of patients. Using flow cytometry analysis, absolute numbers of total $CD3^-CD19^+$ B cells, $CD3^-CD16^+CD56^+$ NK cells, monocytes (figure 2a), of total $CD3^+$ T cells and of $CD3^+CD4^+$ ($CD4^+$) and $CD3^+CD8^+$ ($CD8^+$) subsets (figure 2b) were calculated for healthy subjects and patients at baseline and at 14 days after Campath-1H induction. For the same subjects absolute numbers of monocytes were obtained from a complete blood count done on the same day (figure 2a). Baseline values of total B cells, NK cells, monocytes and T cells and $CD4^+$ and $CD8^+$ T cell subsets of patients in the two groups were not different from those observed in healthy control subjects (n=11; mean age 36 years; range: 32-55), suggesting that the uremic milieu does not alter the composition of circulating PBMC. All the patients included in the study were indeed on chronic hemodialysis from at least 6 months. After Campath-1H induction, B, NK, and T cells in kidney transplant patients were almost completely depleted, consistently with data showing that CD52 is expressed by all these cell populations. On the contrary, depleting effect of Campath-1H on monocytes was much less evident. This phenomenon has been already reported by other authors, and may be due to the reduced expression of CD52 by these cells. (Fig. 2a-b).

When we evaluated more in detail the phenotype of $CD4^+$ T cells spared by Campath-1H induction, we found that the proportion of $CD45RO^+$ cells 14 days after transplant was significantly higher than the one observed in the study patients at baseline, as well as in healthy individuals. CD45RO is the main hallmark of memory T cells and finding that the relative number of cells expressing this marker increased after Campath-1H induction suggests that these cells were more resistant than naïve ones (expressing CD45RA) to its depleting effect (Fig. 2c). Of note, Campath-1H depletion was not affected by the type of maintenance immunosuppressive therapy. The number of circulating lymphocytes at 7 and 14 days after induction was indeed virtually identical in the two groups.

Time-course of repopulating lymphocytes after Campath-1H induction in the two treatment groups

Absolute numbers of repopulating total leukocytes, CD3-CD19+ B cells, CD3-CD16+CD56+ NK cells, monocytes, CD3+ T cells and CD3+CD4+ and CD3+CD8+ T cell subsets in the peripheral blood of patients from baseline to 24 months after transplantation were obtained by FACS analysis.

The number of total circulating leukocytes declined remarkably during the first two weeks after Campath-1H induction, and remained persistently lower than basal levels in both groups of patients up to 2 years after transplant (Fig 3a). At around 6 months after Campath-1H infusion, B cells returned to pre-transplant values (Fig. 3b). Recovery was faster for NK cells that reached baseline values in 2-3 months after transplant (Fig 3c). Depleting effect of Campath-1H on monocytes was transient, as at one month after transplant these cells returned to values recorded at baseline (Fig 3d). Maintenance

therapy with SRL or CsA had no effect on the time of B cell, NK cell and monocyte recovery, although monocytes in SRL patients tended to repopulate more slowly than in patients on CsA (Fig. 3b-d).

Differently from other leukocyte subpopulations, recovery of total T cells was very slow, so that in the SRL group their levels at 24 months after transplant were about one third of the baseline values (Fig 4a). T cells in CsA patients recovered faster, but they required about one year to reach baseline values. In both SRL and CsA-treated patients, the rate of recovery of $CD4^+$ T cells over time was low, to the point that, at month 24 post-transplantation, these cells were approximately one-third of baseline values (Fig. 4b). On the contrary, $CD8^+$ T cells had a significantly different time-course in the two treatment groups. In the SRL patients, at two years after transplant, the number of circulating $CD8^+$ T cells was still only a half of the baseline value. Conversely, $CD8^+$ T cells in CsA patients fully recovered at month 4 and, at one and two years post transplant, their values were twice the baseline ones (Fig. 4c). This resulted into a reduced $CD4^+/CD8^+$ T cell ratio in the CsA group that was significantly lower than preoperatively up to month 24 after transplant (Fig. 4d).

Effect of Campath-1H induction and different maintenance immunosuppression regimens on circulating Treg

Campath-1H depleting effect on circulating Treg

Among $CD4^+$ T cells, those exerting immune regulatory effects are mainly confined in the CD25+ population. However, as $CD4^+CD25^+$ T cells include also effector/memory T cells (Tef), additional markers are needed for the identification of Treg. Recently, evidence came out that the levels of CD25 expression by CD4 Treg and

effector/memory $CD4^+$ T cells is actually different and may be used to discriminate these two cell populations. In particular, $CD4^+$ T cells showing high levels of CD25 have regulatory properties, whereas those whose CD25 expression is low display the characteristics of effector/memory T cells. Resting $CD4^+$ T lymphocytes are CD25 negative [189]. On the basis of this evidence, we used the levels of CD25 marker expression to differentiate $CD4^+CD25^{high}$ Treg, $CD4^+CD25^{low}$ effector/memory T cells, and $CD4^+CD25^-$ resting T cells. Using flow-based frequency enumeration and absolute $CD3^+CD4^+$ cell counts obtained on the same day, absolute numbers of $CD3^+CD4^+CD25^-$, $CD3^+CD4^+CD25^{low}$ and $CD3^+CD4^+CD25^{high}$ cells were calculated in healthy subjects, and in patients at baseline and at 14 days after Campath-1H infusion. The percentage of all these $CD4^+$ T cell subpopulations were similar between healthy subjects and patients at baseline, which further confirms that chronic renal failure does not significantly affect the phenotypic profile of peripheral lymphocytes. Importantly, early after Campath-1H infusion, there was a profound and unselective depletion of $CD4^+CD25^{high}$, $CD4^+CD25^{low}$, and $CD4^+CD25^-$ subsets in all transplant patients, as shown in figure 5a. Within the $CD4^+$ T cell compartment, the percentage of $CD25^{high}$ at 14 days post transplant was comparable to baseline values, confirming that Campath-1H did not spare this cell subset (Fig. 5b). No difference was recorded between the SRL and the CsA groups in the days immediately after Campath-1H infusion.

SRL- but not CsA-based maintenance immunosuppression is associated with in vivo expansion of $CD4^+CD25^{high}$ cells following Campath-1H

As a further step, we aimed to assess whether different maintenance immunouppression might have affected recovery of different $CD4^+$ T cell subpopulations after Campath-1H induction. Among the $CD4^+$ T cell pool, the percentages of $CD25^-$ and $CD25^{low}$ remained relatively stable during the whole follow-up period in both treatment groups. Notably, in patients on SRL, we found a transient decrease in $CD4^+CD25^-$ percentages and a significant mirror increase in $CD4^+CD25^{low}$ cell percentages at 1 month after transplant (Fig. 6a-b), which returned to baseline thereafter. When we looked at the absolute numbers of $CD4^+CD25^-$ and $CD4^+CD25^{low}$, however, both SRL and CsA patients showed a marked depletion of these cell populations after Campath-1H induction that only recovered at two years after transplant. Indeed, at this time point after transplant, $CD4^+CD25^-$ cells were less than one fifth and less than one third of baseline values in SRL and CsA patients, respectively, whereas $CD4^+CD25^{low}$ were still lower than one fourth in the SRL group and around the average of values recorded before Campath-1H administration in the CsA group (Fig. 6e).

From month 1 post-transplant, the percentage of $CD25^{high}$ cell subset within total $CD3^+CD4^+$ T cells progressively increased over baseline in SRL-treated patients (Fig. **6c**), reaching values significantly higher than pre-transplant from month 4 to 24 postoperatively. Conversely, in the CsA group, the trend of $CD4^+CD25^{high}$ cell percentage to increase was milder, so that values significantly higher than pre-transplant were recorded only at month 6 (Fig. 6c). From month 2 up to month 24 after transplant, the percentage of $CD4^+CD25^{high}$ cells was significantly higher in the SRL group than in the CsA group. The same picture was found when we evaluated the absolute numbers of

circulating of $CD4^+CD25^{high}$ cells. Indeed, although the total absolute number of $CD4^+$ T cells was significantly lower at two years after transplant than before in both treatment groups, circulating $CD4^+CD25^{high}$ cells tended to increase, especially in SRL patients (Fig. 6e).

The Treg/Tef ratios were significantly higher ($P<0.05$) in the SRL than in the CsA group from months 2 to 24 (Fig. 6d).

Thereafter, we wondered whether Treg enrichment during lymphocyte recovery was due to an increased proliferation. To address this point, we evaluated the expression of Ki-67, a nuclear protein associated with cell proliferation, in $CD4^+CD25^{high}$ T cells at baseline and at 3-5 months post-transplant, the time of their maximal expansion. Baseline levels of $CD4^+CD25^{high}Ki\text{-}67^+$ T cell were similar between the two patient groups and between patients and healthy subjects, as well as $CD4^+CD25^{low}Ki\text{-}67^+$ and $CD4^+CD25^-Ki\text{-}67^+$ cell percentages (Fig 7). At 3-5 months after transplant, the percentages of $CD4^+CD25^{high}Ki\text{-}67^+$ T cell were significantly higher ($P<0.05$) than at baseline in both treatment groups, but in SRL patients they reached levels significantly higher ($P<0.05$) than in the CsA group. Of note, also $CD4^+CD25^{low}Ki\text{-}67^+$ and $CD4^+CD25^-Ki\text{-}67^+$ cells were found to increase after transplant, but their relative levels were remarkably lower than the ones found in Treg and their values were virtually identical between SRL and CsA patients (Fig 7).

The above findings are in line with an homeostatic expansion of all $CD4^+CD25^+$ T cell populations after Campath-1H induction and either maintenance immunosuppressive therapy. Intriguingly, however, SRL maintenance therapy selectively favoured the expansion of Treg.

Expanding $CD4^+CD25^{high}$ cells express the Treg hallmark FOXP3

The previous results document that Campath-1H-induced T cell depletion favours the emergence of $CD4^+CD25^{high}$ T cells in subjects receiving SRL-maintenance therapy. However, CD25 can not be regarded as a specific hallmark of Treg since, as stated above, also effector/memory T cells express this molecule on their surface. To certainly exclude that our $CD4^+CD25^{high}$ Treg were not recently activated $CD4^+$ T cells, we evaluated their expression levels of CD69, a marker of T cell activation. FACS analysis showed that the large majority (99.0%, range: 98.5-99.5%) of $CD4^+CD25^{high}$ cells from patients in both treatment groups at 2 baseline were $CD69^-$, excluding that they were activated cells.

To ascertain whether the high levels of CD25 expression in regenerating $CD4^+$ cells upon Campath-1H induction reflected a regulatory phenotype, we evaluated the mRNA expression level of FOXP3, a gene that encodes a transcription factor required for Treg development and function and that is now considered the specific marker of Treg. FOXP3 expression was assessed by quantitative real time PCR in $CD3^+CD4^+$ subpopulations of peripheral cells taken at 24 months post-transplant and data were compared with FOXP3 expression in cells from the patients at baseline and from healthy individuals. Using electronically sorted $CD4^+CD25^{high}$, $CD4^+CD25^{low}$, and $CD4^+CD25^-$ cell subsets, we found the highest levels of FOXP3 expression in the $CD25^{high}$ subset both in patients and in healthy individuals, with intermediate and low levels of FOXP3 expression in the $CD25^{low}$ and $CD25^-$ subsets, respectively (Fig. 8a). No difference was observed between patients at baseline in the two randomization arms, nor between patients and healthy subjects, as a further proof that chronic renal failure does not alter the phenotypic profile of these cell populations, at least in the peripheral

blood. At 24 months post-transplant, we found that $CD4^+CD25^{high}$ cells from SRL patients had significantly higher ($P<0.05$) levels of FOXP3 expression as compared with baseline and with patients on CsA at the same time point after transplant (Fig. 8a). Moreover, in order to quantify Treg in the total $CD3^+CD4^+$ cell population, we further evaluated FOXP3 expression in positively selected $CD3^+CD4^+$ T cells. Again, FOXP3 expression was virtually identical between healthy subjects and patients at baseline. Conversely, cells isolated 24 months post-transplant from SRL-treated patients had significantly higher ($P<0.05$) FOXP3 expression compared to $CD3^+CD4^+$ cells from the same patients at baseline or from healthy individuals (Fig. 8b), which is consistent with both $CD4^+CD25^{high}$ T cell expansion observed through flow cytometry and increased FOXP3 expression in this cell subset. Notably, in $CD3^+CD4^+$ T cells of SRL-treated patients, FOXP3 expression was higher than in the CsA group ($P<0.05$) at the same time after transplant (Fig 8b). We found no evidence in any group of significant FOXP3 expression in $CD4^-$ cells (FOXP3 expression less than 0.01 AU).

Altogether these results indicate that, following lymphocyte depletion by Campath-1H induction, SRL but not CsA increased the pool of FOXP3 expressing $CD4^+CD25^{high}$ cells.

CsA- but not SRL-based maintenance immunosuppression is associated with in vivo expansion of $CD8^+CD28^-$ cells following Campath-1H

Beside $CD4^+CD25^{high}$ cells, a distinct population of antigen-primed T cells, characterized by their $CD8^+CD28^-FOXP3^+$ phenotype (Ts) and lack of cytotoxic activity, has been shown to display regulatory functions in human transplant recipients and in a murine autoimmune disease model.

Importantly, the high levels of donor-specific Ts cells have been found in the circulation of organ transplant patients whose immunosuppressive therapy has been successfully reduced without an increased risk of acute rejection [105]. On the basis of this evidence, we evaluated whether Campath-1H induction might have promoted expansion of $CD8^+CD28^-$ cells and whether the two low-dose maintenance immunosuppressive regimens might have differently affected this phenomenon.

As shown in figure 9, the percentage of $CD8^+CD28^-$ cells among $CD8^+$ T cells slightly increased from baseline values in the SRL group but, at 2 years after transplant, they were not significantly different from pre-transplant. Conversely, patients on CsA showed a significant increase in the percentage of $CD8^+$ cells negative for the CD28 marker, to the point that at 2 years after transplant almost all $CD8^+$ were also $CD28^-$.

Expanding $CD8^+CD28^-$ T cells do not express the Treg hallmark FOXP3

As a further step, we wondered whether $CD8^+CD28^-$ T cells isolated from our cohort of patients were also expressing the Treg hallmark FOXP3 gene, in line with the $CD8^+CD28^-$ T suppressor cells described by Cortesini et al. [89, 105]. To this purpose, we evaluated FOXP3 gene expression levels in $CD8^+CD28^-$ T cells from 3 patients randomized to CsA treatment at one year after transplant, using $CD8^+CD28^-$ T cells from 3 healthy subjects as controls. $CD8^+CD28^-$ T cells were electronically sorted from PBMC, and FOXP3 mRNA levels were evaluated thereafter by Real Time PCR. Unexpectedly, we did not find any expression of FOXP3 gene in $CD8^+CD28^-$ T cell neither in patients nor in healthy subjects. Thus, we argued the $CD8^+CD28^-$ T cells that we found expanded after Campath-1H induction in CsA treated patients was different

from the ones described by Cortesini et al. [89, 105]. Thus, we focused subsequent experiments on the better characterized population of $CD4^+CD25^+FOXP3^+$ Treg.

Functional evaluations of Treg cells

T cell alloreactivity in the two treatment groups

Treg are anergic T cells that respond poorly to allogeneic stimuli and are also capable of inhibiting the alloreactive response of effector T cells [87]. Thus, functional assays were performed to address whether the emergence of Treg, generated upon Campath-1H induced lymphopenia, was associated with host T cell hyporesponsiveness against donor antigens. To ascertain the proliferative response of T cells to donor and third-party alloantigens, we used the one way mixed lymphocyte reaction (MLR). In this assay, PBMC of patients were mixed with donor and third party irradiated splenocytes or PBMC (according to the deceased or living type of donor, respectively). The rate of T cell proliferation was assessed by uptake of tritiated thymidine. Moreover, to evaluate the frequency of previously activated/memory T cells, we employed the ELISPOT for IFN-γ following overnight exposure to alloantigens. This test allows to determine the number of previously activated/memory T cell clones by visualization of the IFN-γ product of individual cells.

Samples were taken pre-transplant and at two different time intervals after T cells had repopulated the peripheral blood at adequate amount, *i.e.* at month 12 and 24 post-transplant.

In SRL-treated patients, the anti-donor T-cell proliferative response and the frequencies of IFN-γ producing donor-reactive cells were significantly ($P<0.05$) reduced at both post-transplant points as compared to pre-transplant values (Fig 10a-b). Post-transplant

anti-third party alloreactivity was significantly lower than pre-transplant as well ($P<0.05$) (Fig 10a-b). These results could not be attributed to incomplete recovery of T cell count or to a state of general immunosuppression caused by maintenance therapy, since T cells isolated at the same time points responded normally to a polyclonal T-cell stimulus with phytohemagglutinin (PHA, Fig. 10b). In CsA-treated patients, T cells studied at the same time points showed donor-specific hyporesponsiveness assayed with MLR and ELISPOT compared to pre-transplant (Fig 10a-b) and T-cell response to PHA was normal (Fig 10b).

$CD4^+CD25^{high}$ T cells from SRL-treated patients suppress T cell alloreactivity ex vivo

To clarify the role of Treg in suppressed anti-donor alloreactivity, ELISPOT assays were repeated on PBMC isolated at 24 months post-transplant following $CD4^+CD25^{high}$ cell depletion by sorting. Indeed, in case the reduced immune response against alloantigens relied on active suppression by Treg, their depletion would result in an increased T cell activation. This was exactly what we found in PBMC from SRL patients. Indeed, as shown in Fig 10a, depletion of $CD4^+CD25^{high}$ cells in this group of patients was associated with a statistically significant increase ($P<0.05$) in the frequency of IFN-γ producing effector/memory cells to both donor and third-party antigens. The suppression ratios, defined as frequency after depletion minus frequency before depletion divided by frequency after depletion, were comparable for both anti-donor (0.43 ± 0.06) and anti-third party (0.45 ± 0.06) response. To further confirm that the $CD4^+CD25^{high}$ subset in SRL-treated patients had regulatory activity and to exclude any possible overlapping inhibitory effect of the concomitant immunosuppressive therapy on the function of effector T cells, $CD4^+CD25^{high}$-depleted PBMC obtained from the

SRL patients before surgery were mixed with $CD4^+CD25^{high}$ cells sorted from blood samples of the same patients at 24 months post-transplant. As expected, depletion of naturally occurring $CD4^+CD25^{high}$ Treg resulted in an increased response of baseline PBMC. However, addition of $CD4^+CD25^{high}$ Treg isolated from the same patients at 24 months after transplant restore the pre-depletion levels of response against both donor and third-party antigens (Fig. 11c). Of note, the amount of added Treg did correspond to the percentage of circulating $CD4^+CD25^{high}$ cells in patients pre-transplant, which supports a potential clinical significance of the above findings.

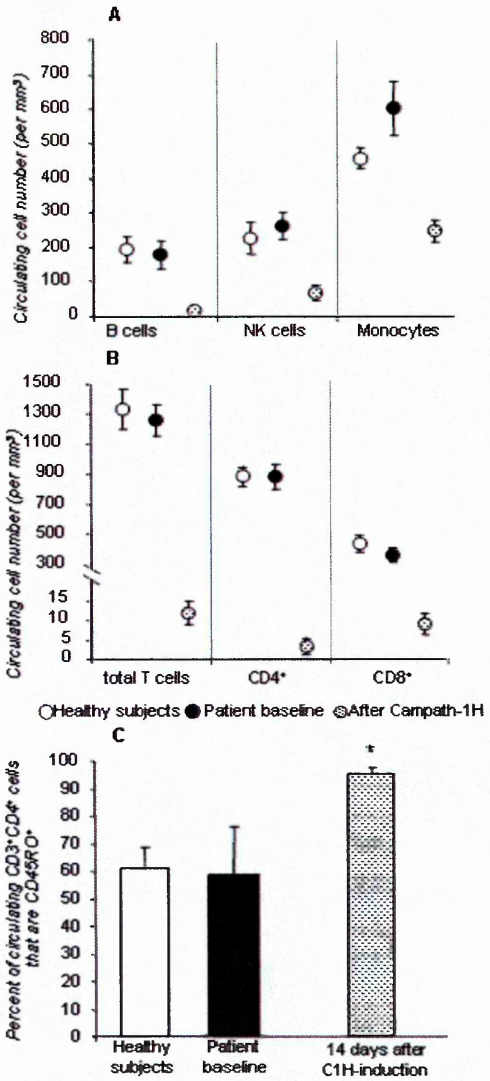
On the other hand, in patients on maintenance CsA therapy, $CD4^+CD25^{high}$ cell depletion had no effect on the frequencies of anti-donor IFN-γ producing T cells taken at 24 months post-transplant (Fig. 11b), suggesting that Treg did not play a significant role in the hyporesponsiveness to donor alloantigens in CsA-treated patients. Thus, we wondered whether the reduced response against donor antigens in the PBMC of these patients relied on an alternative mechanism, such as cell anergy. To address this hypothesis, we added progressively increasing concentrations of IL-2 to PMBC isolated from CsA patients at 24 months post transplant and exposed to donor or third-party alloantigens in the ELIPOT assay. Indeed, IL-2 is able to restore cell activity in anergic cells. As shown in figure 11d, higher concentrations of IL-2 were indeed able to increase the IFN-γ frequencies against donor antigens to reach anti-third party values (Fig. 11d). Thus, anergy rather than regulation seemed to contribute to donor-specific hyporesponsiveness in CsA-treated patients.

Tables and figures

Table 1. Baseline patients' characteristics according to the randomization arm.

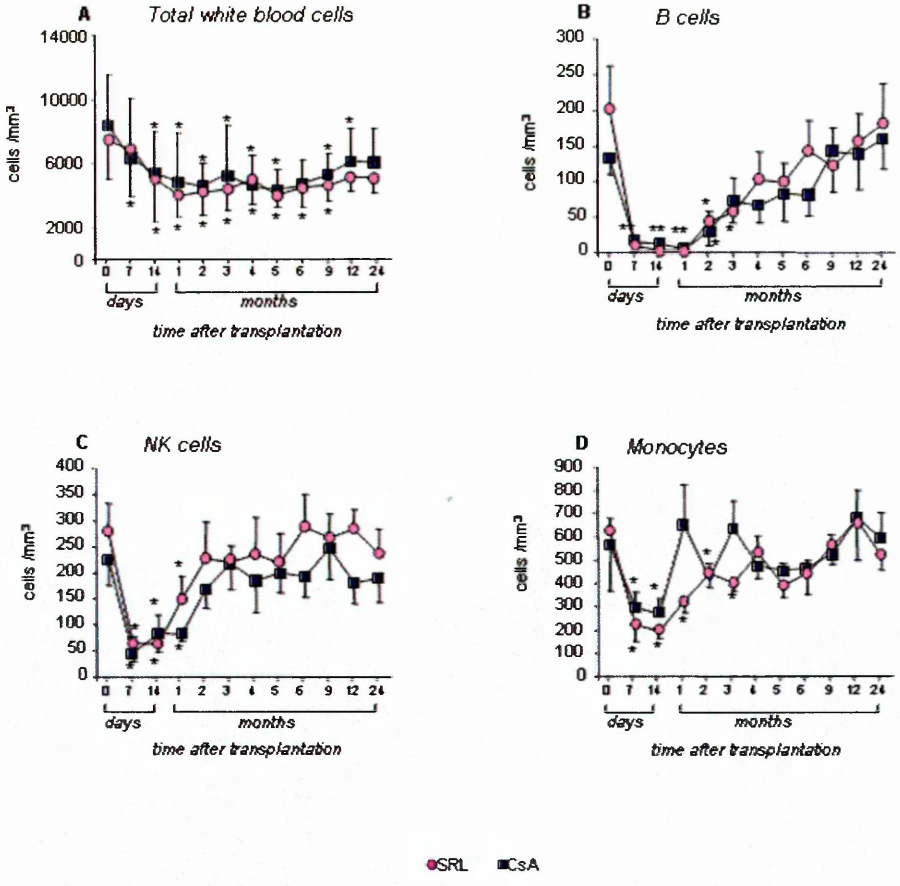
	SRL *(n=11)*	CsA *(n=10)*
Donors		
Age (years)	47.5 ± 16.0	42.3 ± 13.2
Gender (M/F)	7/4	5/5
Weight (Kg)	79.5 ± 10.0	72.0 ± 13.5
Type of donor		
Cadaveric	11	8
Living	0	2
Cold ischemia time (h)	16.7 ± 2.3	14.6 ± 3.7
Warm ischemia time (min)	31.3 ± 7.7	27.1 ± 5.3
Recipients		
Age (years)	53.2 ± 8.9	47.0 ± 16.5
Gender (M/F)	6 / 5	7 / 3
Weight (Kg)	71.2 ± 10.1	74.3 ± 16.9
Mismatches		
A	1.6 ± 0.5	1.2 ± 0.6
B	1.6 ± 0.7	1.4 ± 0.7
DR	1.2 ± 0.6	1.0 ± 0.8
Cause of renal failure		
Polycystic kidney disease	1	2
Membranous nephropathy	1	0
IgA nephropathy	3	3
Interstitial inflammation	1	0
Pyelonephritis	3	0
Glomerulonephritis	1	0
Unknown	1	5

Data are mean ± SD.



Effect of Campath-1H (C1H), on circulating leukocyte subsets in renal transplant patients. Using flow cytometry analysis, absolute numbers of total $CD3^-CD19^+$ B cells, $CD3^-CD16^+CD56^+$ NK cells, monocytes (a), of total $CD3^+$ T cells and of $CD3^+CD4^+$ ($CD4^+$) and $CD3^+CD8^+$ ($CD8^+$) subsets (b) were calculated for healthy subjects (n=11), participant patients at baseline (before kidney transplantation, n=21) and at 14 days after transplantation and Campath-1H induction (n=21, data from patients receiving either sirolimus, SRL, or cyclosporine A, CsA, maintenance therapies combined with mycophenolate mofetil, MMF, were cumulated since no difference was recorded between the two groups at this time). (c) Percent of memory $CD45RO^+$ cells within the $CD3^+CD4^+$ subset from healthy subjects, patients at baseline and at 14 days after Campath-1H induction (cumulative data from the SRL and the CsA group).

Figure 2

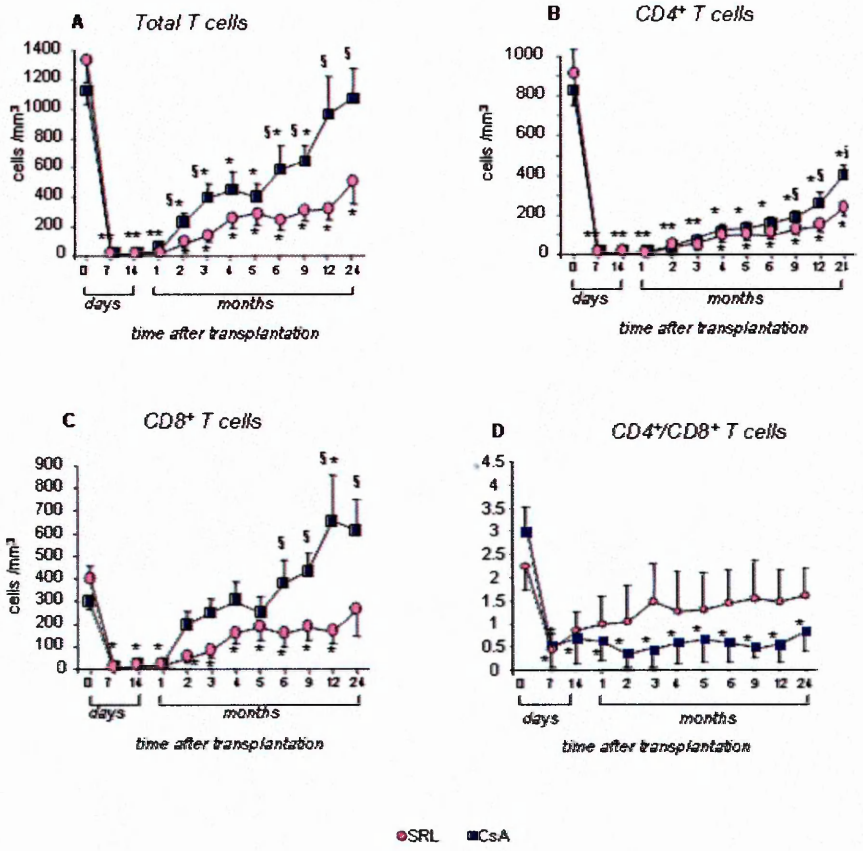


Time course of circulating leukocyte subsets in renal transplant patients after Campath-1H (C1H) induction

Kinetics of absolute numbers of repopulating total leukocytes (a), $CD3^{-}CD19^{+}$ B cells (b), monocytes (c), and $CD3^{-}CD16^{+}CD56^{+}$ NK cells (d), $CD3^{+}$ T cells in the peripheral blood of Campath-1H-treated renal transplant patients from baseline (pre-transplant, time 0) to 24 months after transplantation.

Data from recipients of SRL plus MMF maintenance therapy are pink circles (n=11, n=10 at 24 months), data from recipients of CsA plus MMF are blue squares (n=10). Data are mean ± s.e.m. *P<0.05 vs. pre-transplant (time 0), §P<0.05 vs. SRL-treated group at the same time point.

Figure 3

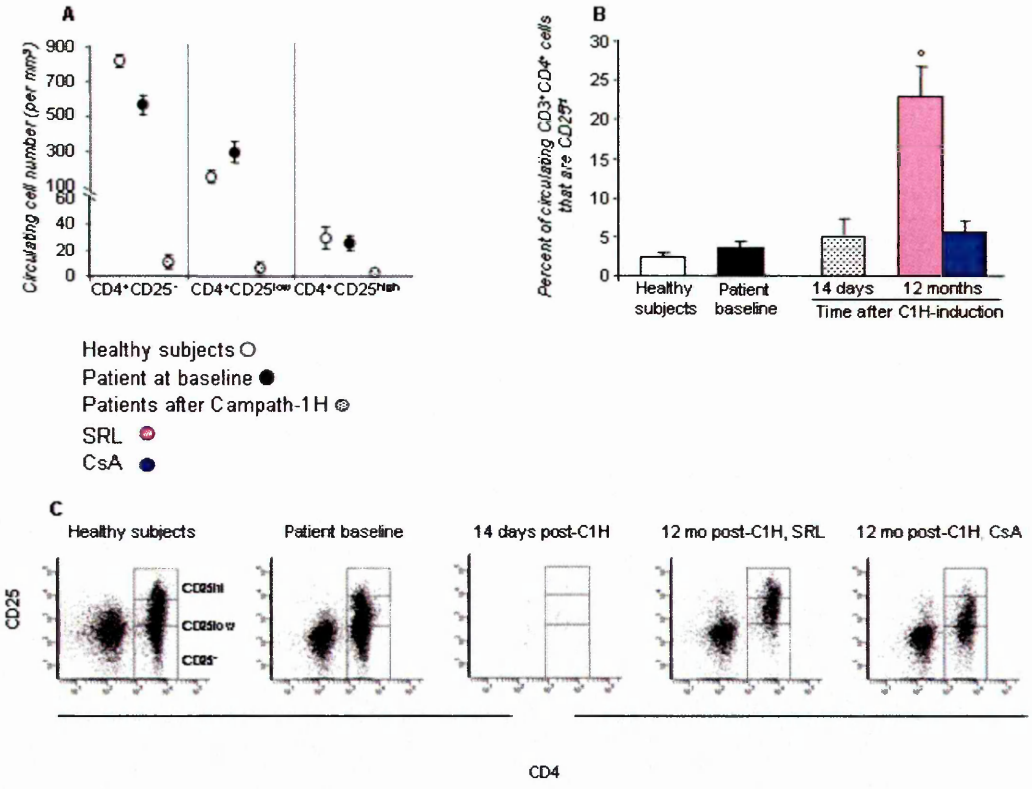


Time course of circulating T lymphocyte subsets in renal transplant patients after Campath-1H (C1H) induction

Kinetics of absolute numbers of repopulating total $CD3^+$(a), $CD3^+CD4^+$ (b), $CD3^+CD8+$ (c) T cells, and $CD4^+/CD8^+$ ratio (d) in the peripheral blood of Campath-1H-treated renal transplant patients from baseline (pre-transplant, time 0) to 24 months after transplantation.

Data from recipients of SRL plus MMF maintenance therapy are pink circles (n=11, n=10 at 24 months), data from recipients of CsA plus MMF are blue squares (n=10). Data are mean ± s.e.m. *P<0.05 vs. pre-transplant (time 0), §P<0.05 vs. SRL-treated group at the same time point.

Figure 4

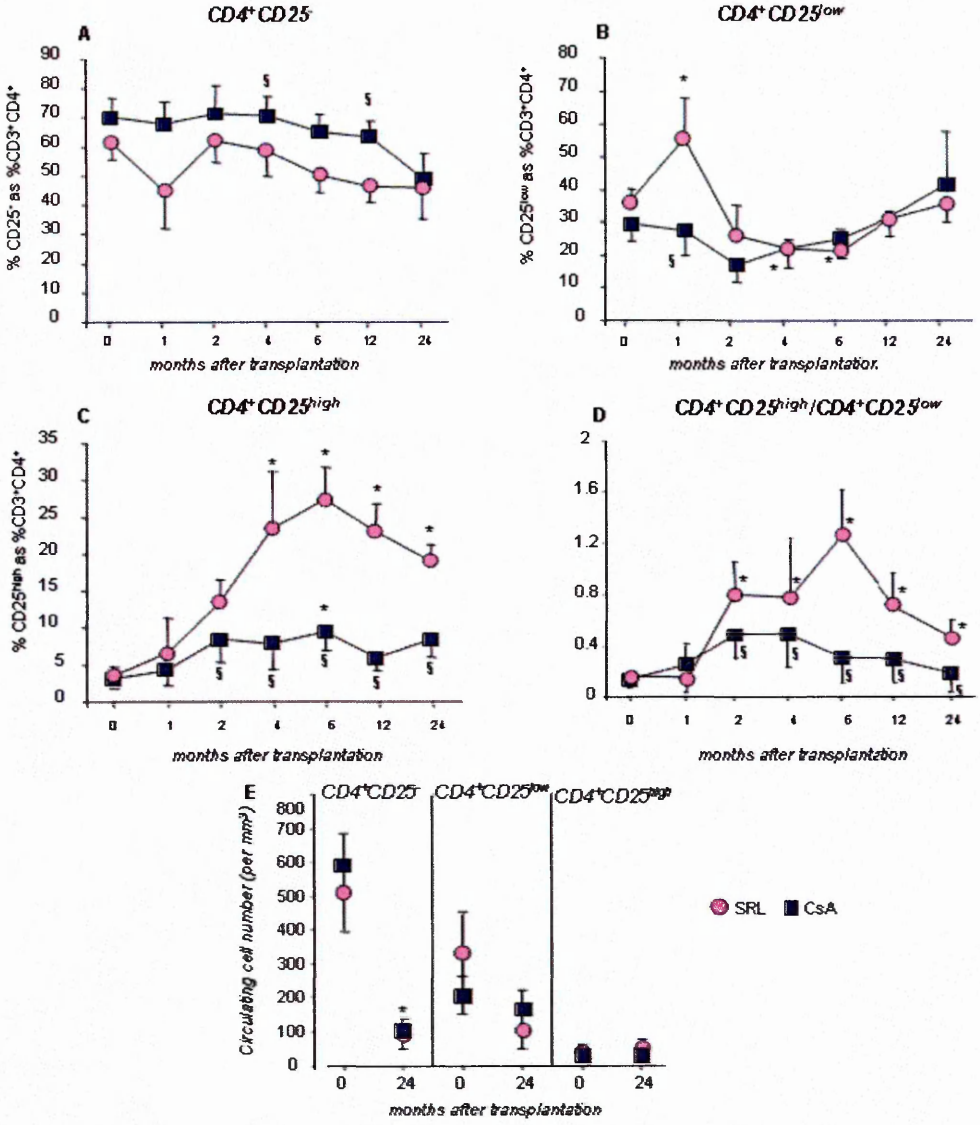


Effect of Campath-1H on different CD3⁺CD4⁺CD25⁺ T cell populations

(a) Using flow-based frequency enumeration and absolute CD3⁺CD4⁺ cell counts obtained on the same day, absolute numbers of CD3⁺CD4⁺CD25⁻, CD3⁺CD4⁺CD25^{low} and CD3⁺CD4⁺CD25^{high} cells were calculated in healthy subjects (n=11), in patients at baseline (n=21) and at 14 days post transplant (after Campath-1H, n=21). Campath-1H profoundly depleted all CD3⁺CD4⁺CD25⁻, CD3⁺CD4⁺CD25^{low} and CD3⁺CD4⁺CD25^{high} cell subsets. (b) Percent of CD25^{high} within the CD3⁺CD4⁺ subset from healthy subjects (n=11), patients at baseline (n=21), at 14 days (n=21, data from the SRL and the CsA groups were cumulated since no difference was recorded between the two groups at this time) and at 12 months (data from the SRL, n=11, and the CsA, n=10 groups, are presented separately) after transplantation and Campath-1H (C1H-induction). Data are mean ± s.e.m. °P<0.05 vs healthy subjects, patient baseline and CsA.

(c) Representative FACS plots of CD25 expression on CD3⁺CD4⁺ cells from healthy subjects, patients at baseline, at 14 days post-transplant (post-C1H) and in patients on either SRL or CsA therapy studied at 12 months post-transplant. CD3⁺CD4+ cells were designated CD25⁻ if CD25 expression fell within the background staining observed using an isotype control, CD25^{high} if CD25 expression exceeded that seen in the CD4⁻ population, and CD25^{low} if CD25 expression fell between these regions.

Figure 5

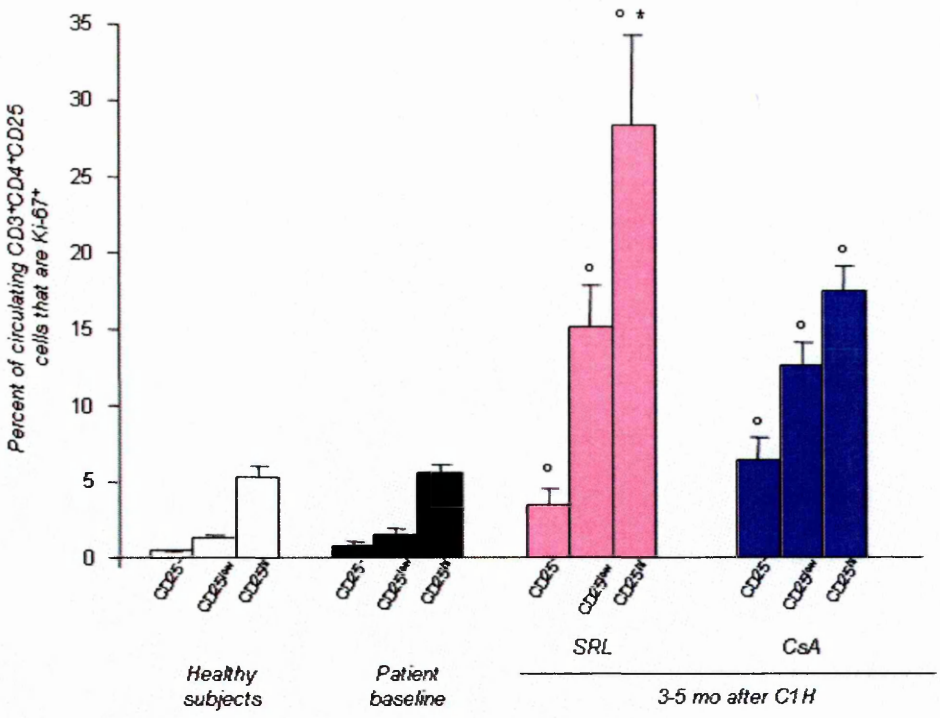


SRL maintenance therapy favours expansion of $CD4^+CD25^{high}$ subset following Campath-1H induction

Using the gating shown in panel Figure 5 (panel c), post-transplant longitudinal analysis of the percent of $CD3^+CD4^+$ that were $CD25^-$ (a), $CD25^{low}$ (b) or $CD25^{high}$ (c) are shown. Panel d shows the ratio between $CD3^+CD4^+$ $CD25^{high}$ and $CD3^+CD4^+$ $CD25^{low}$, whereas in panel e are shown data on absolute numbers of $CD3^+CD4^+CD25^-$, $CD3^+CD4^+CD25^{low}$, and $CD3^+CD4^+CD25^{high}$ at baseline and at 24 months after Campath-1H infusion.

Data from recipients of SRL plus MMF maintenance therapy are pink circles (n=11, n=10 at 24 months), data from recipients of CsA plus MMF are blue squares (n=10). Data are mean ± s.e.m. *P<0.05 vs. pre-transplant (time 0), §P<0.05 vs. SRL-treated group at the same time point.

Figure 6

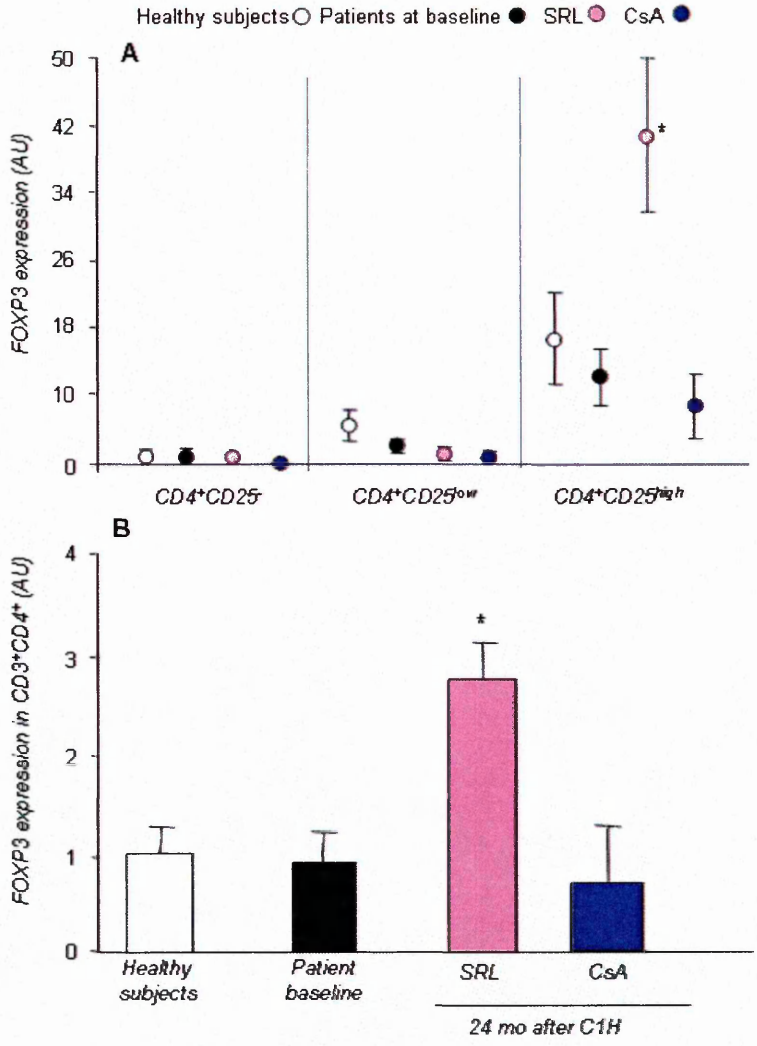


Expression of the proliferation marker Ki-67 in $CD3^+CD4^+CD25^{high}$ cells

Expression of the proliferation marker Ki-67 in $CD3^+CD4^+CD25^{high}$ cells from patients at baseline and at 3-5 months post-transplant (at the time of maximal expansion of $CD3^+CD4^+CD25^{high}$ cells) and in healthy subjects (n=6 for each group).

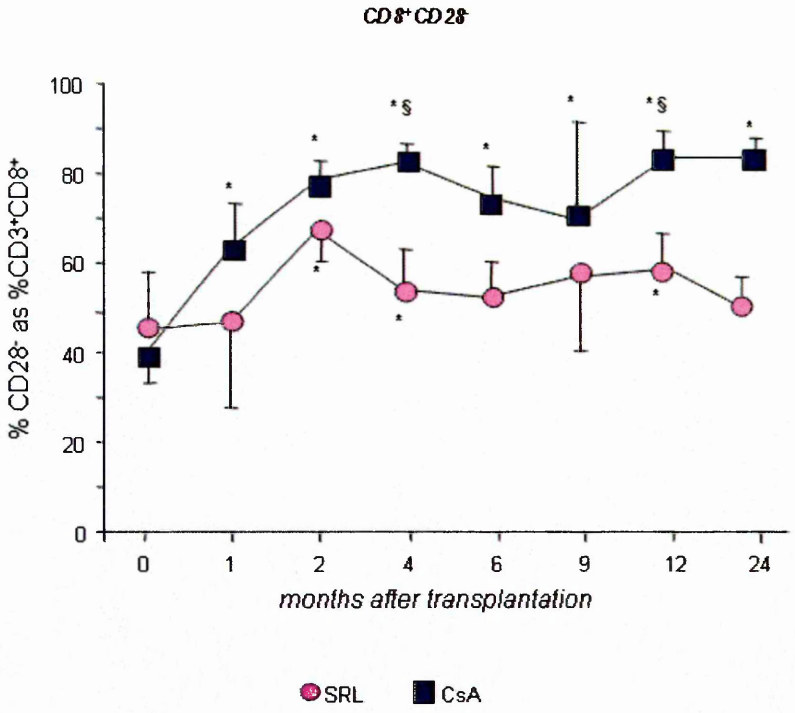
Data are mean±s.e.m. *P<0.05 vs. healthy subjects, patients at baseline and CsA-treated patients at the same time point.

Figure 7



FOXP3 expression in cells from renal transplant patients following Campath-1H induction. (a) FOXP3 mRNA is predominantly expressed in $CD3^+CD4^+CD25^{high}$ cells in all groups studied, with significantly higher FOXP3 in $CD4^+CD25^{high}$ versus $CD4^+CD25^-$ and $CD4^+CD25^{low}$ samples both in patients and in healthy subjects. FOXP3 expression in $CD3^+CD4^+CD25^{high}$ cells at 24 months was significantly higher in SRL-treated (n=10, pink circles) as compared to CsA-treated (n=10, blue circles) patients, patients at baseline (n=21) and healthy subjects (n=11). (b) FOXP3 expression was measured in positively selected $CD3^+CD4^+$ cells in healthy subjects (n=11), in patients at baseline (n=21) and at 24 months after transplantation (SRL, n=10, pink bars, CsA, n=10, blue bars). SRL maintenance therapy was associated with higher FOXP3 expression in $CD3^+CD4^+$ cells as compared with patients at baseline, patients on CsA and healthy subjects. FOXP3 mRNA expression was measured by quantitative real-time PCR. Results are expressed as AU, taking the expression in $CD3^+CD4^+$ cells from a pool of healthy subjects as calibrator (AU=1).

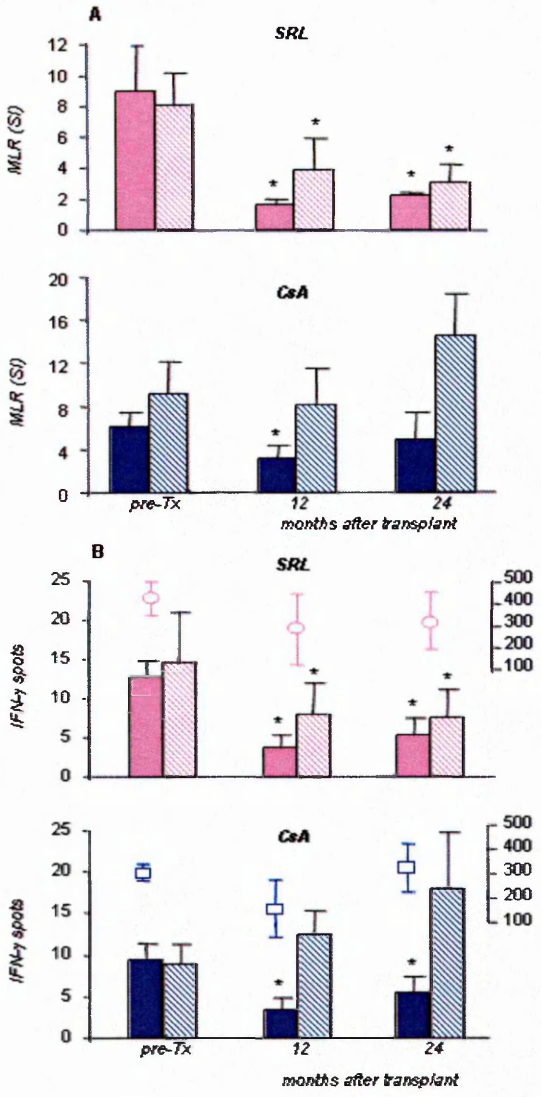
Figure 8



Time course of CD8+CD28- circulating T lymphocytes in renal transplant patients after Campath-1H (C1H) induction

The percentage of $CD3^+CD8^+$ cells negative for CD28, respectively, at different time points after transplant. Data from recipients of SRL plus MMF maintenance therapy are pink circles (n=11, n=10 at 24 months), data from recipients of CsA plus MMF are blue squares (n=10). Data are mean ± s.e.m. *P<0.05 vs. pre-transplant (time 0), §P<0.05 vs. SRL-treated group at the same time point.

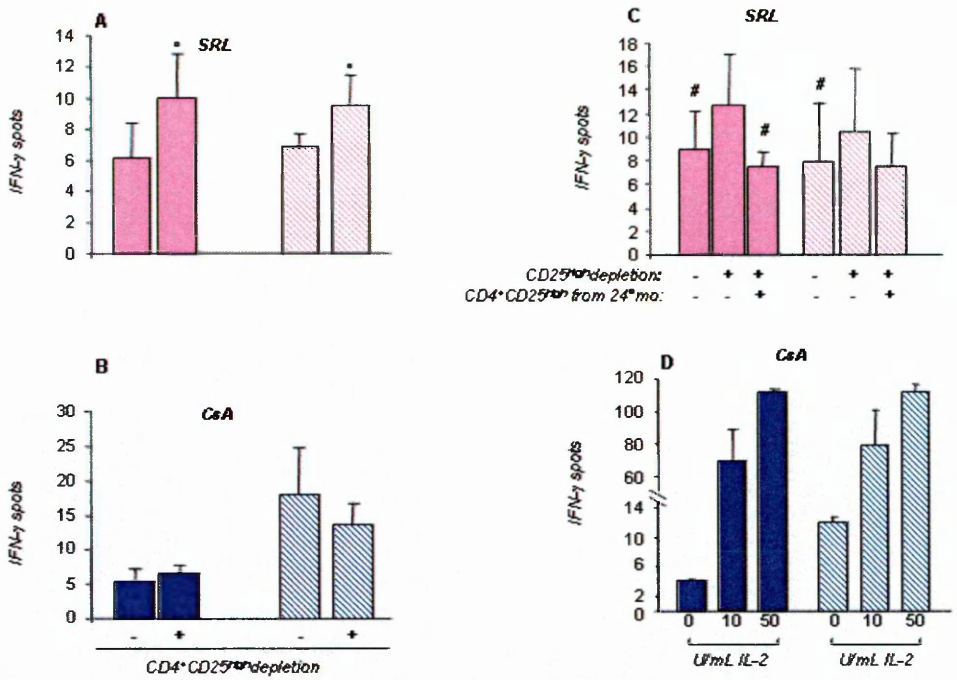
Figure 9



Functional assays on repopulating T cells following Campath-1H induction in renal transplant patients.

(a) Proliferative responses (expressed as stimulation index, SI) to donor (closed bars) and third-party (hatched bars) antigens in 6 days mixed lymphocyte reaction (MLR) of T cells from transplant patients receiving SRL (n=6 pink colour) or CsA (n=6 blue colour) for whom donor cells were available. Each subject was studied at baseline and at 12 and 24 months post-transplant. (b) Frequency of previously activated/memory T cells by ELISPOT for IFN-γ following overnight exposure to donor (closed bars) and third-party (hatched bars) alloantigens. The frequencies of IFN-γ specific T cells were plotted as spots/300,000 PBMC from transplant patients receiving SRL (n=6 pink colour) or CsA (n=6 blue colour). Each subject was studied at baseline and at 12 and 24 months post-transplant. The responses to phytohemagglutinin (PHA, 10 μg/ml), used as positive controls are shown as circles.

Figure 10



Functional evaluation of CD4$^+$CD25^{high} Treg in SRL patients and response to IL-2 in the CsA group

(a-b) Effect of CD4$^+$CD25^{high} depletion by FACS sorting on the frequencies of IFN-γ producing cells. The number of spots/300,000 PBMC measured before (-) and after (+) CD4$^+$CD25^{high} depletion in PBMC from renal transplant patients studied 24 months after surgery are shown. CD4$^+$CD25^{high} depletion increased the frequencies of anti-donor (closed bars) and anti-third party (hatched) IFN-γ producing cells in the SRL (pink bars, n=6) but not in the CsA group (blue bars, n=6). (c) Addition (+) of autologous CD4$^+$CD25^{high} to CD4$^+$CD25^{high}-depleted (+) PBMC isolated at baseline, reduced the frequencies of anti-donor (closed bars) and anti-third party (hatched bars) IFN-γ producing T cells. CD4$^+$CD25^{high} cells were isolated by sorting from SRL patients at 24 months after transplantation (n=3) and 5,000 cells were added to 300,000 pre-transplant CD4$^+$CD25^{high}-depleted PBMC from the same subjects.

(d) IL-2 reverted donor-specific hypo-responsiveness in CsA-treated patients. The frequencies of IFN-γ producing cells in response to donor (closed bars) and to third-party antigens (hatched bars) in PBMC from CsA-treated patients studied 24 months post-transplant (n=3) were evaluate in the absence (0) or in the presence of 10 or 50 U/mL of IL-2.

Data are mean ±s.e.m. *P<0.05 vs. pre-Tx. °P<0.05 vs. pre-depletion (-), #P<0.05 vs. CD25high-depleted PBMC (+/-).

Figure 11

Discussion

In this part of the project, we showed that Campath-1H induction was associated with a profound and long-lasting lymphocyte depletion in kidney transplant patients and that phenotypic and functional characteristics of repopulating cells were affected by different maintenance immunosuppressive regimens. In particular, SRL – but not CsA - therapy was associated with the expansion of functionally active $CD4^{+}CD25^{high}FOXP3^{+}$ Treg. Importantly, however, all Campath-1H treated patients displayed a reduced alloreactivity against donor antigens at 2 years after transplant independently from maintenance immunosuppressive regimen.

Effect of Campath-1H induction on peripheral leukocytes: extent of depletion and time-course of recovery in kidney transplant recipients on low-dose SRL or CsA, both combined with low-dose MMF as maintenance immunosuppression

In our series of 21 kidney transplant recipients, a single 30 mg infusion of Campath-1H was well tolerated and induced a profound and long-lasting depletion of T lymphocytes and, to a lower extent, of B cells, NK, and monocytes. The milder depleting effect on the latter subsets was in line with other reports and possibly relies on the lower expression levels of the CD52 antigen by these cell populations.

The ideal dose of Campath-1H as induction therapy in renal transplantation has still not yet been identified. Previous studies used higher doses or repeated administrations of the antibody, with the attempt of inducing more sustained lymphocyte depletion [126]. However, here we clearly showed that a single 30 mg infusion of Campath-1H is enough to deplete lymphocytes for around 9-12 months in kidney transplant patients,

suggesting that this regimen should be preferred to other ones employing higher doses, in order to reduce potential adverse events and saving costs.

Intriguingly, the depleting effect of Campath-1H on T cells was preferentially targeted toward the naïve population, suggesting that the memory cell counterpart may have a higher resistance to the antibody. Indeed, at 14 days after treatment, the proportion of T cells showing the CD45RO memory marker was significantly higher than at baseline. The obvious reason why different leukocyte subsets were differentially susceptible to Campath-1H might rely on the expression levels of CD52 receptor. Indeed, it has been clearly shown a correlation between high levels of expression of CD52 on T cells, in particular naïve T cells, and the intensity of depletion achieved when Campath-1H was administered [190].

This is consistent with Pearl's data [173], who found that in renal transplant recipients who received induction with Campath-1H/deoxyspergualin and no maintenance immunosuppression, T cells repopulating after depletion were predominantly activated memory-like T cells, which expanded in the first month after transplantation. These memory-like T cells, which are prone to activate an immune response, were the prevalent T cell population in the blood and in the allograft during rejection episodes [173], that uniformly occurred in all five patients included in that study. Conversely, in our trial, maintenance immunosuppression with MMF combined with either SRL or CsA was probably instrumental to limit the immune response of memory T cells after Campath-1H induction. Indeed, MMF has been shown to inhibit memory cell proliferation both *in vitro* in human mixed lymphocyte cultures [191] and *in vivo* in F5 TCR transgenic mice exposed to the TCR specific NP68 peptide [192].

Maintenance immunosuppression with either SRL or CsA did not affect the repopulation of B, NK cells and monocytes after Campath-1H induction, which was completed within 6 months after transplant in both treatment groups. In both groups, $CD4^+$ T lymphocytes had a slow and incomplete recovery, whereas $CD8^+$ T cell reappearance in the periphery occurred significantly slower in SRL than in CsA patients. In SRL patients, indeed, $CD8^+$ number at two years after transplant was still only a half of the baseline value. Conversely, $CD8^+$ T cells in CsA patients fully recovered at month 4 and at one and two years post transplant their values were twice the baseline ones. This resulted in a prolonged inversion of the $CD4^+/CD8^+$ ratio, which remained significantly lower than at baseline up to two years after transplant in the CsA group.

The phenomenon of $CD4^+/CD8^+$ inversion has been already described in patients undergoing lymphocyte depletion with polyclonal [193, 194] antibodies followed by CNI-based therapies. Little is known about the process of T-cell regeneration and lymphocyte lifespan *in vivo* [195]. Apart from the persistent depletion of the $CD4^+$ cells, a disproportionate regeneration in the $CD8^+$ cell subset may occur. This is in line with findings showing that T-cell differentiation in the adult is primarily extrathymic and seems to be predominantly $CD8^+$ [196]. Interestingly, a recent paper showed that, even after Campath-1H induction, recovery of $CD8^+$ T cells was much faster than that of $CD4^+$ T cells in patients given MMF and tacrolimus as maintenance immunosuppression [174]. Moreover, most of these cells were $CD8^+CD28^-$, in line with our findings in CsA treated patients. Importantly, $CD8^+CD28^-$ cells have been described as immunosenescent $CD8^+$ T cells, i.e. terminally differentiated and non-proliferating cells, that, upon reaching the limit number of cell division, do not die but rather survive

as expanded T clones. Expanded $CD8^+CD28^-$ T cells may compete for the immune space with $CD4^+$ T cells, suppressing their proliferation and therefore delaying $CD4^+$ T cell-recovery after Campath-1H induction.

Effect of Campath-1H induction and different maintenance immunosuppressive regimens on circulating Treg

Polyclonal and monoclonal anti-T cell antibodies have been used as an integral part of tolerance induction in experimental transplantation and clinical trials [122]. Their immunosuppressive activity has been though to result primarily from profound T cell depletion from the circulating pool via complement-dependent lysis or Fas/Fas ligand-mediated apoptosis [197], although emerging evidence suggests that expansion of Treg may have a role [198]. Following ALS-induced lymphopenia in C57/BL6 mice [199], the reduction of $CD4^+CD25^+$ T cells number was smaller than that of $CD4^+CD25^-$ cell subset, which raised the idea that Treg may be resistant to ALS-mediated depletion. At variance with ALS, Campath-1H does not selectively spare Treg since it induced a profound and unselective depletion of CD4+CD25-, $CD4^+CD25^{low}$ and $CD4^+CD25^{high}$ subsets in all renal transplant patients included in the present study. These results are consistent with previous findings that all CD4+ T-cell subsets, including CD4+CD25+, express at similar densities on their cell surface the CD52 target antigen of Campath-1H and addition of Campath-1H to human blood *in vitro* caused depletion of $CD4^+CD25^{high}$ cells [200].

After Campath-1H induced depletion, however, Treg repopulation significantly differed between patients receiving SRL or CsA as maintenance immunosuppression. Indeed, the percentage of $CD25^{high}$ cell subsets progressively increased over baseline in SRL-

treated patients, reaching values significantly higher than pre-transplant from month 4 to 24 postoperatively. Conversely, the percentage of $CD25^{high}$ cells among $CD4^+$ T cells in the CsA group had only a transient increase at 6 months post-transplant, and remained substantially unchanged thereafter compared with pre-transplant values. Intriguingly, $CD4^+CD25^{high}$ expansion in SRL patients was anticipated by a spike of $CD4^+CD25^{low}$ activated T cells at month 1 after transplant. Theoretically, this immune activation might have created a favourable milieu to promote Treg expansion as a counterbalancing mechanism to turn off the inflammatory response. However, this is just a speculative hypothesis.

Our findings are in line with the already reported increase of $CD4^+CD25^+$ T cells in peripheral lymphoid organs from rats treated with **SRL**. Moreover, Battaglia et al. recently described that $CD4^+CD25^+FOXP3^+$ Treg expanded ex vivo in the presence of SRL and prevented rejection of [beta]-islet transplants *in vivo* [177]. SRL also induces de novo expression of FOXP3 in murine alloantigen-specific T cells dose dependently, which appeared to be TGF-β1 dependent [201]. Because SRL can induce the expression of TGF-β1, it may be an important mechanism contributing to the development of antigen-specific Treg [202]. Interestingly, a recent study suggests that SRL can induce regulatory functions in conventional $CD4^+$ T cells in culture [113]. Furthermore, evidence suggests that **SRL**-conditioned dendritic cells are poor stimulators of allogenic T cells but enrich for antigen-specific Treg, which can prolong cardiac graft survival in mice [203].

$CD4^+CD25^{high}$ T cell expansion in our patients on SRL therapy may result from two mutually nonexclusive and possibly complementary mechanisms. First, Campath-1H-induced lymphopenia may promote the selective homeostatic proliferation of naturally

occurring $CD4^+CD25^{high}$ cells. This phenomenon has been reported in IL-2-treated genetically lymphopenic *Rag*$^{-/-}$ mice upon adoptive transfer of $CD4^+CD25^+$ cells, whereas exogenous $CD4^+CD25^-$ cells proliferated poorly [204]. Second, Campath-1H-induced lymphopenia may stimulate the expansion of $CD4^+CD25^-$ cells followed by their conversion into $CD4^+CD25^{high}$ cells, as observed in other studies in immunodeficient [205] mice. Interchange between $CD4^+CD25^-$ and $CD4^+CD25^+$ phenotype has been also observed in immunocompetent wild type mice following T cell depletion with ALS [206] and in ALS-stimulated human PBMC *in vitro* [198]. Finding a selective increased expression of the proliferation marker Ki-67 in $CD4^+CD25^{high}$ cells from renal transplant patients after Campath-1H, would support the first hypothesis. However, on the basis of these data, we can not exclude that proliferating $CD4^+CD25^{high}$ cells derived from the conversion of $CD4^+CD25^-$ into $CD4^+CD25^+$ cells. Moreover, other studies are needed to asses if an increase in number of naïve Treg, i.e. an increase in thymic output, could have contributed to the observed accumulation of $CD4^+CD25^{high}$ in SRL –treated patients.

According to our findings, a recent paper by Knechtle's group [175] reported that $CD4^+CD25^+FOXP3^+$ regulatory T cells increased in kidney transplant patients after immunodepletion with Campath-1H and maintenance therapy with SRL. Interestingly, these Authors suggested that peripheral expansion of Treg may not fully explain their increase, thus they argued that also de novo generation/expansion from $CD4^+CD25^-$ T cells may be involved.

On the other hand, lack of development of $CD4^+CD25^{high}$ Treg with CsA could be explained by the fact that IL-2 - whose generation is inhibited by CsA - promotes acquisition of CD25 molecules [118] and is a surviving factor for Treg *in vitro* [118]

and *in vivo* [207]. This interpretation is in line with data showing that IL-2 therapy given during immune reconstitution to pediatric patients with sarcoma increases the frequency and absolute numbers of Treg compared with patients who did not receive IL-2 [204]. Finding that CsA, by inhibiting calcineurin phosphatase-dependent NF-AT translocation into the nucleus [208], suppresses FOXP3 promoter activity, mRNA and protein expression in T cells [208], is also consistent with the present *in vivo* data.

Our data are in line with those of a recent study showing that kidney transplant patients receiving CNI maintenance treatment had a significantly lower percentage of peripheral $CD4^{+}CD25^{high}$ T cells compared with patients receiving SRL [114]. Recently, evidence has been provided that conversion of kidney transplant recipients from tacrolimus/MMF to SRL monotherapy, significantly increased the percentage and absolute number of circulating $CD4^{+}CD25^{high}FOXP3^{+}$ Treg [113]. These data support the concept that SRL promotes Treg expansion, which in our cohort resulted into a significantly higher Treg/Teff ratios than in the CsA group during the whole follow-up period.

Importantly, we carefully characterized the phenotype of circulating Treg using all the most important markers. Indeed, they did not express the CD69 activation marker and expressed high levels of FOXP3. Notably, the expression levels of FOXP3 in $CD4^{+}CD25^{high}$ T cells from SRL treated patients were significantly higher than those found in the CsA group, and this might be associated with a higher immune regulatory effect. Moreover, as we will discuss in the next paragraph, these cells were capable to suppress the alloreactive immune response of autologous effector $CD4^{+}CD25^{-/low}$ T cells against donor antigens in co-cultures.

Functional evaluations of circulating lymphocytes

As a final step, we evaluated the functional characteristics of circulating lymphocytes, with a particular attention to $CD4^{+}CD25^{high}FOXP3^{+}$ Treg. *In vitro* assessment of lymphocyte alloreactivity has been claimed as a potential tool to shape immunosuppressive therapy and to predict the risk of acute rejection. Moreover, it might allow understanding how Campath-1H induction efficiently prevents acute rejection with lower than conventional doses of maintenance immunosuppression.

Our experiments on T cell alloreactivity focused on the direct pathway of allorecognition. Alloreactive T cells recognize HLA-mismatched tissue *via* two different pathways: in the direct pathway, responder T cells recognize intact foreign MHC-peptide complexes on the surface of donor antigen-presenting cells (APC) [26]. In the indirect pathway, T cells recognize donor allopeptides on self-MHC molecules after having been processed and presented by recipient APC. During the past few years, the relevance of both pathways of antigen allorecognition for long-term graft outcome has controversially been discussed. Priming by the direct pathway has classically been associated with the early posttransplantation period and especially with acute rejection, because professional donor APC are present during the first months only. In contrast, priming by the indirect pathway was suggested to play a main role in the long term; therefore, indirectly primed T cells were considered key mediators for chronic immune-mediated graft injury [26]. However, recent studies suggest that both pathways may persist and be of relevance for interstitial fibrosis and tubular atrophy. Notably, Herrera *et al.* demonstrated how recipient dendritic cells, when co-cultured with allogeneic dendritic cells or endothelial cells, can acquire substantial levels of allogeneic MHC-peptide complexes and subsequently prime T cells by both allorecognition pathways

[209]. Hence, early directly primed T cells could be maintained and reactivated by graft cell populations such as graft endothelial cells but also by recipient dendritic cells at later time points. A very recent study evaluating the impact of both direct and indirect reactivity against donor antigens has recently shown that direct hyporesponsiveness against donor antigens was the only variable significantly correlated with graft function at multivariate analysis [159]. Thus, our evaluation of the direct alloreactivity was expected to provide a strong instrument to assess immune activation against HLA antigens.

In our trial, the post-transplant T cell proliferative response against donor antigens and the frequencies of IFN-γ producing donor-reactive lymphocytes were significantly reduced as compared to pre-transplant values in both patient groups included. Importantly, this could not be attributed to incomplete recovery of T cell count, or to a state of general immunosuppression due to maintenance therapy, since T cells isolated at the same time points responded normally to a polyclonal T cell stimulus with phytohemagglutinin. A reduced immune reactivity against donor antigens, in the setting of an otherwise preserved immune response is a hallmark of tolerance [46]. Thus, the present finding provides the evidence that after Campath-1H induction, kidney transplant recipient may develop signs of tolerance. Although hyporesponsiveness toward donor antigens might have been affected by ongoing immunosuppression, it suggests that grafts from Campath-1H treated patients might be protected from chronic immune injury, which may improve their long-term outcomes.

We also evaluated reactivity of PBMC toward third party (TP) cells, harvested from subjects who had a number of mismatches for HLA similar to the one between the donor and recipient. Intriguingly, SRL patients showed a reduced response also against

these cells, whereas those on CsA had a substantially unaltered reactivity as compared with baseline. This phenomenon might reflect the alloantigen cross-reactivity of TCR from expanding Treg in SRL patients [210], or alternatively, a phenomenon of bystander regulation [211].

Our results are in line with the ones by Trzonkoski et al. [174] showing that, in Campath-1H treated patients receiving MMF and the tacrolimus as maintenance immunosuppression, the response to donor alloantigens was suppressed, whereas the one toward mismatched third-party alloantigens recovered partially with the time after transplantation.

As a final step, we asked which mechanisms were at the basis of the different *in vitro* immune responses in the two treatment groups. To this purpose, we depleted $CD4^{+}CD25^{high}$ taken at 24 months after surgery from total PBMC, to assess the impact of these cells in the overall immune response. In SRL patients, depletion of these cells resulted into a significant relapse of alloreactivity against both donor and third party antigens, suggesting that these cells were actually crucial in maintaining hyporesponsiveness toward donor alloantigens. Moreover, the addition of Treg taken at 24 months after transplant to baseline PBMC resulted in a hyporesponse against donor antigens, further confirming the immune regulatory function of these cells.

Conversely, depletion of Treg did not affect response against donor or TP antigens from CsA patients. This was not an unexpected finding, as the proportion of Treg after transplantation did not significantly change as compared to baseline in the CsA group. On the other hand, increasing concentrations of IL-2 were associated with progressively higher proliferative responses of PBMC against both donors and TP antigens. Importantly, after IL-2 pulsing, the number of spot against donor cells equalled that

observed against TP antigens. This is consistent with an anergic state of circulating leukocytes against donor antigens in CsA treated patients.

Anergy is another mechanism of immunological tolerance which, however, might be theoretically less powerful and stable than regulation [212]. Indeed, high levels of IL-2, such as those that may be associated with immune system activation during infections, may break this tolerance status. Importantly, in transplant patients receiving calcineurin inhibitors, maintenance of the anergic state seems directly dependent on IL-2 pathway inhibition [212]. Thus, restore of normal IL-2 levels after CsA tapering/withdrawal might eventually be associated with breakdown of the tolerance state.

A still unanswered question of our study is whether expansion of $CD8^+CD28^-$ T cells in the CsA group might have played a role in the donor-specific hyporesponsiveness of PBMC isolated from these patients. $CD8^+CD28^-$ T cells have been described as a population with regulatory properties, which directly interacts with antigen-presenting cells rendering them tolerogenic by inducing the downregulation of costimulatory molecules and upregulation of the inhibitory receptors immunoglobulin-like transcripts (ILT)3 and ILT4 [89]. Importantly, this population has been found positive for the FOXP3 regulation marker. According with this evidence, we tested whether the $CD8^+CD28^-$ T cells isolated from our CsA treated patients showed an increased expression of this gene. As these cells showed no expression of FOXP3, we argued that they were not responsible for immune regulation. However, this cannot be definitely excluded and would require *ad hoc* experiments to formally assess this hypothesis. Indeed, a recent paper showed that in renal transplant recipients given Campath-1H as induction and MMF plus tacrolimus as maintenance immunosuppression, CD8+CD28- T cells were the main repopulating population [174], which displayed the capability to

suppress the proliferation of $CD4^+$ T cells. In this paper, however, no data on FOXP3 expression in CD8+CD28- T cells have been provided.

Thus, the first part of the study clearly described the phenotype and functional characteristics of lymphocytes emerging after Campath-1H induction in renal transplant patients receiving maintenance immunosuppression with either SRL or CsA, both combined with MMF. In the second part, we aimed at assessing the clinical counterpart of the different lymphocytic phenotype and functional profile in the two treatment groups.

RESULTS (II Part)

2. Long-term clinical outcomes in renal transplant patients receiving Campath-1H induction and low-dose SRL or CsA, both combined with low-dose MMF, as maintenance therapy: does the number of circulating Treg matter?

Introduction

In the previous part of the study we showed that the CD52 targeted monoclonal antibody Campath-1H, via lymphocyte depletion, allowed a subset of $CD4^+CD25^{high}FOXP3^+$cells with regulatory activity to emerge, provided that maintenance immunosuppression with sirolimus (SRL), but not the calcineurin-inhibitor cyclosporine (CsA), added on to low-dose mycophenolate mofetil (MMF) is used as background steroid-free maintenance therapy. These findings indicate that Campath-1H and the combination of low-dose SRL and MMF would create the ideal environment for T-cell regulation to occur. Increased Treg would favor graft acceptance, and lowering the immune response of the host against the allograft might translate into a reduced incidence of acute rejection in the short-term and, possibly, of chronic rejection in the long period.

An immune modulatory role of $CD4^+CD25^+$ Treg in the setting of transplantation was first provided by in a rat bone marrow transplantation model involving the adoptive transfer of transplantation tolerance [213]. Since then, there has **been** a growing body of data in the literature indicating that $CD4^+CD25^+$ Treg maintain dominant transplantation tolerance, and $CD4^+CD25^+$ T cells from an animal tolerised to an allograft by many immunosuppressive regimens could transfer donor-specific transplant tolerance to a

naïve animal [78]. Importantly, $CD4^+CD25^+$ Treg could be found inside the tolerised graft, and these cells can have both direct and indirect allospecificity for donor antigens [78].

However, despite significant advances in understanding the development, function, and therapeutic efficacy of Treg in certain well-defined rodent models of organ transplantation, the relevance of Treg in the clinical setting remains unclear. Recent data showed that in kidney biopsies of transplant recipients with borderline acute changes the Treg/T cytotoxicity cell infiltrating ratio was higher than in biopsies with acute rejection [214]. Others have reported in patients with no history of acute rejection, *ex vivo* regulation of the host immune response toward the mismatched HLA-DR allopeptides by peripheral blood Treg [157]. Thus, although far from conclusive, these findings would suggest that Treg may limit episodes of acute rejection also in humans. Moreover, the presence of Treg in per protocol biopsies of renal transplant recipients has been reported to discriminate harmless from injurious infiltrates, evidenced by independently predicting better graft function 2 and 3 yr after transplantation [159].

On the other hand, a recent study in 83 renal transplant biopsies, found that Foxp3 mRNA was higher in grafts with rejection than in grafts without signs of rejection [215] and Foxp3 did not correlate with favorable graft outcomes, even when the analysis was restricted to biopsies with rejection.

To assess whether Treg may indeed protect from the development and progression of chronic allograft injury, we compared long-term changes in graft structure and function - as assessed by serial GFR and proteinuria evaluations and per protocol graft biopsies - in the two cohorts of renal transplant recipients originally randomized to SRL or CsA-based therapy that eventually did or did not show an increase in circulating Treg cells.

Specific aims

In the second part of the study, we aimed to assess whether the impact of diverse maintenance immunosuppressive regimens on peripheral lymphocyte phenotype and function and on Treg expansion translated into different clinical outcomes in kidney transplant recipients given Campath-1H as induction therapy.

More in detail, specific aims of this second part of the project were:

i) To compare graft survival and function (evaluated as serum creatinine, measured GFR and RPF, and 24h/proteinuria) during the follow-up period between kidney transplant patients who received induction therapy with Campath-1H and maintenance immunosuppression with low-dose SRL or CsA, both combined with low-dose MMF;

ii) to compare the histology score at the kidney graft per-protocol biopsy at 2 years after transplant between patients in the two randomization arms;

iii) to compare blood pressure levels and metabolic parameters in the two treatment groups;

iv) to compare the incidence of acute rejection episodes in the two low-dose maintenance immunosuppression after Campath-1H induction;

v) to assess the safety profile of Campath-1H induction associated with low-dose SRL or CsA, both combined with low-dose MMF as maintenance immunosuppression;

vi) to evaluate whether a relationship exists between the levels of circulating Treg at 2 years and graft function or histology changes in the per-protocol biopsy performed at the same time after transplantation.

Results

Graft survival and function

One patient in the SRL group died at 18 **months** due to sepsis and one additional patient, with post-DGF severe renal insufficiency, lost his graft 25 months post transplant. No patient on CsA died or lost the graft throughout the observation period. From the transplant day up to month 2 post-transplant, mean serum creatinine levels similarly decreased in both groups (Figure 12). Then, they progressively increased in SRL-treated patients, while remained relatively stable in those on CsA (Figure 12). Nevertheless, there was no significant difference in serum creatinine concentrations between the two cohorts at any time-point after transplant. Thereafter, we evaluated the slopes over time of both GFR and RFP that had been measured every 6 months. According with the trend of serum creatinine levels, GFR decline from month 6 post-transplant to study end tended to be faster on SRL than on CsA (SRL -2.92±0.33, CsA -0.28±0.44 ml/min/1.73 m^2 per year) (Figure 13). Notably, the renal function decline for CsA patients was even lower than the one reported for healthy subjects after 40 years of age, *i.e.* -1 ml/min/1.73 m^2 per year. Time dependent changes in RPF showed a similar trend (SRL -10.80±5.45, CsA -1.86±3.09 ml/min/1.73 m^2 per year) (Figure 13). At month 6 post-transplant, SRL patients had a significantly higher GFR compared to CsA ones. However, differences in GFR values between cohorts at subsequent visits were never significant, as well as differences in RPF that never achieved the statistical significance at any considered time point after transplant (Table 2).

During the observation period, 6 of the 11 SRL patients compared to 4 of the 10 CsA patients developed proteinuria persistently higher than 0.5 g/24h. At 24 months post transplant, urinary protein excretion rate in SRL and CsA group was 0.93±1.03 and

0.77±0.92 g/24 h, respectively. No significant difference in 24-h proteinuria was observed between the two cohorts at any considered follow-up evaluation (Figure 14).

Histology

Seven patients in the SRL and 6 in the CsA groups consented to per-protocol biopsy at 2 years post-transplant.

All samples from SRL patients showed mild to moderate tubular atrophy, interstitial fibrosis with focal interstitial inflammation and arteriosclerosis consistent with chronic allograft injury (Figure 15 and Table 3). In five cases light microscopy also showed mild increase in mesangial cells and matrix (Figure 15) and in one additional sample, (V.G.) mild increases in mesangial cells and matrix, focal marginated glomerular intercapillary leukocytes, mild endothelial cell proliferation and incipient segmental sclerosis in one glomerulus were suggestive of a transplant glomerulitis. Since this pattern was associated with a high SRL trough level (14.6 ng/ml), significant renal function deterioration and proteinuria, a SRL-related glomerulopathy was diagnosed, and the patient was changed to CsA maintenance immunosuppression, though proteinuria remained persistently elevated.

Only 3 of the 6 samples from CsA-treated patients showed moderate to severe interstitial fibrosis. Mild to moderate glomerular abnormalities were also seen in 4 patients, mainly characterized by mesangial hypercellularity (Figure 15) and, in one case, mild increase in mesangial matrix. In this patient, recurrent IgA nephropathy was diagnosed after immunofluorescence analysis of biopsy specimens.

Thereafter, we scored histology lesions according with CADI grading and, as shown in Table 3, we found that mean CADI score was numerically, although not significantly, higher in SRL- than in CsA-treated patients.

C4d glomerular staining was similar between the two study groups, whereas tubular staining was significantly higher ($P<0.01$) in the SRL- than in the CsA- group (Table 3).

Other laboratory and clinical outcomes and safety profile

On follow-up, blood pressure control and lipid profile were similar in the two cohorts, although patients on SRL tended to have higher levels of triglycerides and total cholesterol (Table 4). Three patients, one on SRL and two on CsA had acute rejection episodes at 14, 9 and 210 days post-transplant, respectively, that fully recovered with intravenous methylprednisolone. Of note, in one rejecting patient CsA blood levels were below the recommended target (Table 5). Three SRL and two CsA patients developed DGF, defined as dialysis requirement within the first week after transplant.

Fever of unknown origin, potentially associated with lymphocytolysis after Campath-H induction, occurred in both treatment arms, but was significantly more frequent in CsA patients (3 vs. 7 patients in the SRL vs. CsA group, respectively. $P<0.05$). Four cytomegalovirus reactivations, without clinical disease, arose in 4 patients on CsA and recovered with gancyclovir therapy. Two herpes zoster virus reactivations were observed in two patients on SRL and recovered with acyclovir therapy. Four bacteria pneumonia were diagnosed in 3 SRL- and 1 CsA- treated patient. There were also 2 *E. coli* septic episodes per group (most likely originating from the urinary tract) and 1 acute cholangitis in the SRL group. All the above bacteria infections recovered with

antimicrobial therapy. One patient per group was hospitalized because of congestive heart failure and 1 on SRL because of an acute coronary event (Table 5).

Thus, after Campath-1H induction, the safety/efficacy profile of a maintenance regimen including either low-dose SRL or low-dose CsA, both combined with low-dose MMF was similar. Indeed, the increased Treg pool associated with SRL therapy did not significantly affect either the risk of rejection, or the risk of infections.

Immunosuppressive drug monitoring

As shown in Table 6, mean SRL and CsA trough levels were within the target range (trough blood concentrations of 5–10 ng/ml for SRL and from 120 to 220 ng/ml in the first month post-transplant, and from 70 to 120 ng/ml thereafter for CsA) throughout the whole follow-up period. The mean blood levels of SRL were very stable, whereas those of CsA tended to progressive decrease during the follow-up period. Indeed the CsA dosing was progressively lowered with the attempt to use the minimal dose required to prevent acute rejection. Of note, the areas under the time-concentration curves (AUC) at different time points show that exposure of these patients to study drugs was lower than the one usually followed in standard immunosuppressive regimens.

Three SRL- and 7 CsA- treated patients with fever attributed to alemtuzumab (n=7) or MMF dose reduction because of diarrhea or CMV-associated leukopenia (n = 3), transiently received low-dose corticosteroids in addition to their current maintenance immunosuppressive therapy.

Peripheral blood Treg cell counts

As reported in the previous chapter of this thesis, pre-transplant T lymphocyte counts were similar in the two groups. From month 2 post-transplant, following the profound T cell depletion induced by alemtuzumab, $CD3^+CD4^+$ T cells slowly recovered in both cohorts but remained low over baseline up to 24 months after transplantation (Figure 4b).

The proportion of circulating $CD4^+$ cells that comprised $CD25^{high}$ cells (Treg cells) were comparable in both groups pre-transplant (Figure 6c). From month 1 post transplantation, the percentage of $CD25^{high}$ cell subset in total $CD3^+CD4^+$ T lymphocytes progressively increased in SRL group, whereas in CsA-treated patients the tendency of the $CD4^+CD25^{high}$ cell percentage to increase was milder. Thus, at month 12 post transplant, the percentage of Treg in patients given SRL were approximately four-fold and, at 24 months, twice higher the values than in those on CsA (Figure 6c).

We evaluated the presence of any potential relationship between the percentage of circulating Treg at 1 year and clinical outcomes. There was no significant correlation between percentage of Treg in total $CD3^+CD4^+$ T lymphocytes at 24 months post-transplant and CADI scores, GFR decline and 24-h proteinuria at last visit in the study group as a whole, as well as in each cohort considered independently.

Post hoc analysis of patient outcomes according to the levels of circulating Treg

In a *post-hoc* analysis, we stratified patients according to the Treg counts at 2 years of follow-up above (n=10) or under (n=11) the median value. Among patients with higher Treg, 8 were on SRL and 2 on CsA therapy, whereas 3 patients on SRL and 8 on CsA therapy had lower Treg levels (Table 7). Gender distribution, donor and recipient age at transplant, cold and warm ischemia time, HLA –A, B and DR mismatches, and the total

number of HLA mismatches ($Treg^+$: 4.2±1.1; $Treg^-$: 3.9±1.6) were similar in the two cohorts.

No significant difference in serum creatinine levels was observed between the two cohorts at any considered follow up evaluation (Figure 16). As shown in Table 8, there was a not statistically significant trend toward higher histology injury among $Treg^+$ patients. The incidence of adverse events between the two cohorts was similar (Table 9). Three patients, 1 in the $Treg^+$ and 2 in the $Treg^-$ had acute rejection episodes at 14, 9 and 210 days post-transplant, respectively, that fully recovered with intravenous methylprednisolone.

Tables and figures

Table 2. GFR and RPF throughout the follow-up period in the two treatment

Months	0	6	12	18	24	30
Measured GFR *(ml/min/1.73 m^2)*						
SRL		64.9±10.9*	65.6±16.1	59.8±17.1	61.8±17.0	58.6±15.2
CsA		49.6±14.4	53.5±13.4	52.7±17.2	56.3±18.7	49.1±16.7
RPF *(ml/min/1.73 m^2)*						
SRL		543.2±209.1	516.5±199.9	382.0±91.5	438.1±64.5	415.1±158.0
CsA		426.3±101.3	416.4±132.4	406.4±129.2	405.6±125.4	336.4±127.1

Data are mean+SD. *P<0.05

Table 3. Histology score in the per-protocol biopsies at 2 years after transplant.

Patients	CADI	Tubular C4d	Glomerular C4d
SRL			
1. VG	5	1.5	1.0
2. FG	8	1.5	1.8
3. PC	2	2.0	1.8
4. ZB	5	1.0	0.5
5. GG	9	0.5	0.4
6. CR	6	0.5	0.5
7. TG	4	0.7	0.2
Mean	5.6	1.1*	0.9
Median	5.0	1.0	0.5
CsA			
1. RS	5	0.5	0.3
2. TA	2	0	0.3
3. GM	1	0	0.5
4. NF	5	0.5	0.5
5. CM	9	0	0.7
6. GG	0	0	2.3
Mean	3.7	0.2	0.8
Median	3.5	0	0.5

*P<0.01 vs. CsA

Table 4. Arterial blood pressure and systemic biochemical parameters throughout the follow-up period in the two treatment groups.

Months post-	0	6	12	18	24	30
SBP *(mmHg)*						
SRL	136±17	142±17	146±8	136±15	146±9	150±16
CsA	143±20	132±16	145±18	154±18	147±17	152±11
DBP *(mmHg)*						
SRL	77±12	78±10	85±11	79±17	85±8	85±11
CsA	86±9	79±6	94±11	92±4	92±9	91±13
Total cholesterol *(mg/dL)*						
SRL	236±46	248±56	243±58	235±36	240±34	237±45
CsA	225±46	223±45	209±32	210.4±18	217±22	208±35
HDL cholesterol *(mg/dL)*						
SRL	66±22	57±16	49±9	57±13	53±11	60±16
CsA	48±18	46±14	55±15	45±14	51±11	49±12
Triglycerides *(mg/dL)*						
SRL	174±89	151±85	173±134	141±77	138±52	155±93
CsA	212±89	183±89	148±64	152±65	172±78	154±79

Data are mean+SD.

Table 5. Patients with adverse events in the two treatment arms.

	SRL (n=11)	CsA (n=10)
Delayed graft function	3	2
Acute rejection	1	2
Viral infection		
CMV	0	4
HZV	2	0
Bacterial infection		
Pneumonia	3	1
Urinary Tract	2	0
Sepsis	1	1
Acute colangitis	1	0
Congestive Heart Failure	1	1
Ischemic coronary disease	1	0

Table 6. Immunosuppressive drug dosing, trough levels, and AUC throughout the study period in the two treatment arms.

SRL

Time Post-Tx	SRL dose *(mg/day)*	SRL trough *(ng/mL)*	SRL AUC_{0-24} *(ng*h/mL)*
Month 1	4.3 ± 1.3	7.8 ± 3.2	
Month 3	4.4 ± 1.9	8.6 ± 2.7	
Month 6	3.9 ± 1.5	9.1 ± 3.7	307 ± 100
Month 12	4.0 ± 0.9	9.3 ± 2.3	332 ± 73
Month 18	3.6 ± 1.1	10.9 ± 5.3	306 ± 52
Month 24	3.4 ± 1.0	11.0 ± 4.1	318 ± 62
Month 30	3.7 ± 1.7	8.5 ± 1.4	290 ± 78

CsA

Time Post-Tx	CsA dose *(mg/day)*	CsA C_0 *(ng/mL*	CsA AUC_{0-12} *(ng*h/mL)*
Month 1	308 ± 72	164 ± 69	
Month 3	238 ± 60	102 ± 22	
Month 6	225 ± 49	120 ± 32	3328 ± 757
Month 12	195 ± 62	87 ± 32	2817 ± 570
Month 18	194 ± 79	92 ± 27	2712 ± 626
Month 24	164 ± 64	79 ± 26[1,2]	2404 ± 765[2]
Month 30	143 ± 61[1]	75 ± 42[1,3]	2169 ± 806[3]

Data are mean±SD. [1]$p<0.01$ vs month 1; [2]$p<0.05$ vs month 6; [3]$p<0.01$ vs month 6

Table 7. Donor and recipient parameters according to Treg counts at one year after transplant. $Treg^+$ and $Treg^-$ are patients with levels of Treg higher or lower than the median value, respectively.

	$Treg^+$ *(n=10)*	$Treg^-$ *(n=11)*
Donors		
Age (years)	50.7 ± 11.4	39.8 ± 15.7
Gender (M/F)	5/5	7/4
Weight (Kg)	76.0 ± 11.8	76.3 ± 12.8
Type of donor		
Cadaveric	10	9
Living	0	2
Cold ischemia time (h)	16.8 ± 2.4	15.0 ± 3.8
Warm ischemia time (min)	30.1 ± 6.0	28.1 ± 7.8
Recipients		
Age (years)	53.2 ± 8.9	47.0 ± 16.5
Gender (M/F)	6 / 5	7 / 3
Weight (Kg)	75.8 ± 12.4	69.8 ± 14.3
Therapy		
SRL	8*	3
CsA	2*	8
Mismatches		
A	1.5 ± 0.5	1.4 ± 0.7
B	1.5 ± 0.7	1.5 ± 0.7
DR	1.2 ± 0.6	1.0 ± 0.8

Data are mean ± SD or median (range).*$p<0.01$ vs $Treg^-$

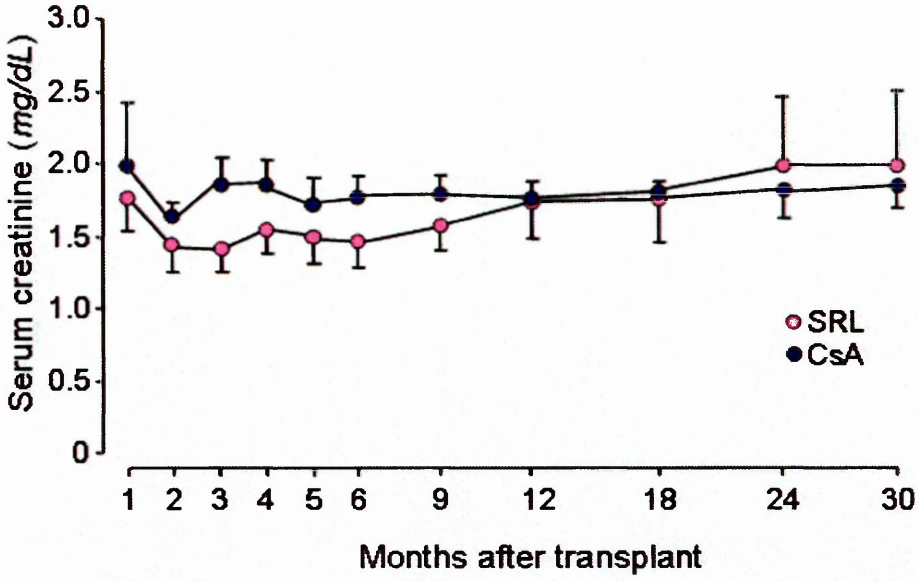
Table 8. Histology score (CADI) and C4d expression in graft biopsies from renal transplant recipients according to Treg count.

Patients	CADI	Tubular C4d	Glomerular C4d
***Treg*$^{+}$**			
1. VG	5	1.5	1.0
2. PC	2	2.0	1.8
3. ZB	5	1.0	0.5
4. GG	9	0.5	0.4
5. CR	6	0.5	0.5
6. TG	4	0.7	0.2
Mean	*5.2*	*1.0*	*0.7*
Median	*5.0*	*0.9*	*0.5*
***Treg*$^{-}$**			
1. RS	5	0.5	0.3
2. TA	2	0	0.3
3. GM	1	0	0.5
4. NF	5	0.5	0.5
5. CM	9	0	0.7
6. GG	0	0	2.3
7. FG	8	1.5	1.8
Mean	*4.3*	*0.4*	*0.9*
Median	*5.0*	*0*	*0.5*

Table 9. Patients with adverse events according to randomization to Treg counts.

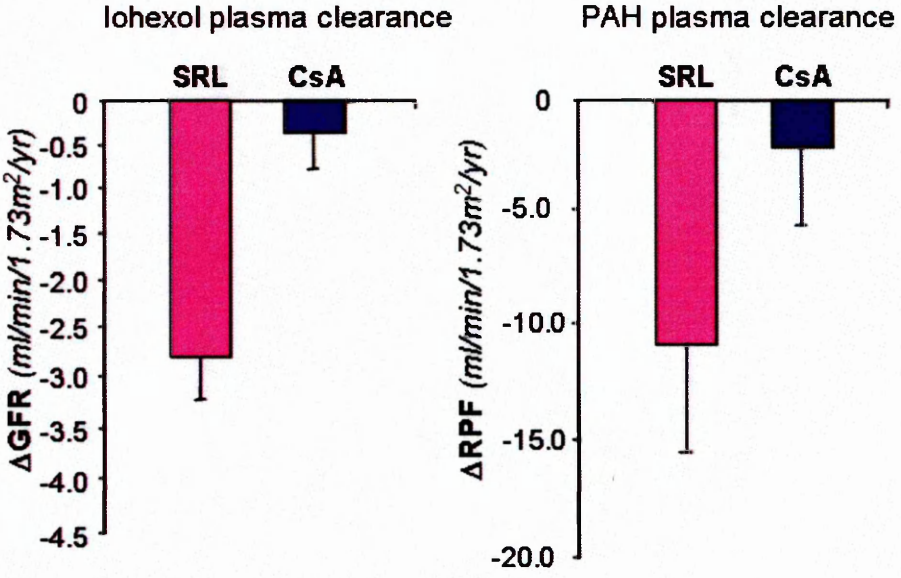
	$Treg^+$ *(n=10)*	$Treg^-$ *(n=11)*
Delayed graft function	3	2
Acute rejection	1	2
Viral infection		
CMV	0	4
HZV	2	0
Bacterial infection		
Pneumonia	3	1
Urinary Tract	1	1
Sepsis	1	1
Acute colangitis	1	0
Congestive Heart Failure	1	1
Ischemic coronary disease	1	0

Data are number (%).



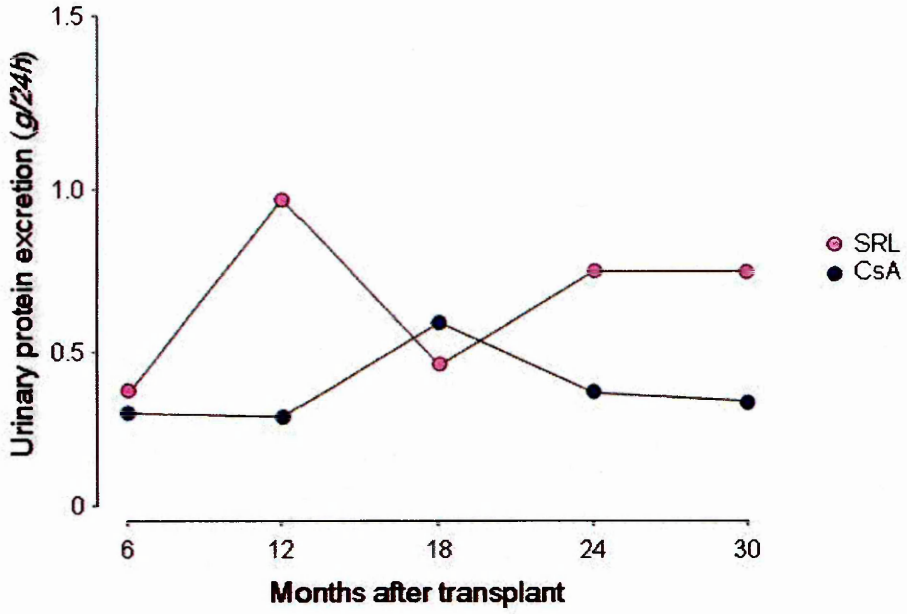
Graft function in the two treatment groups
Serum creatinine levels at different time points after transplant in the SRL (pink circles, n=11, n=10 at 24 months) and CsA (blue circles, n=10) group. Data are mean ± SE.

Figure 12



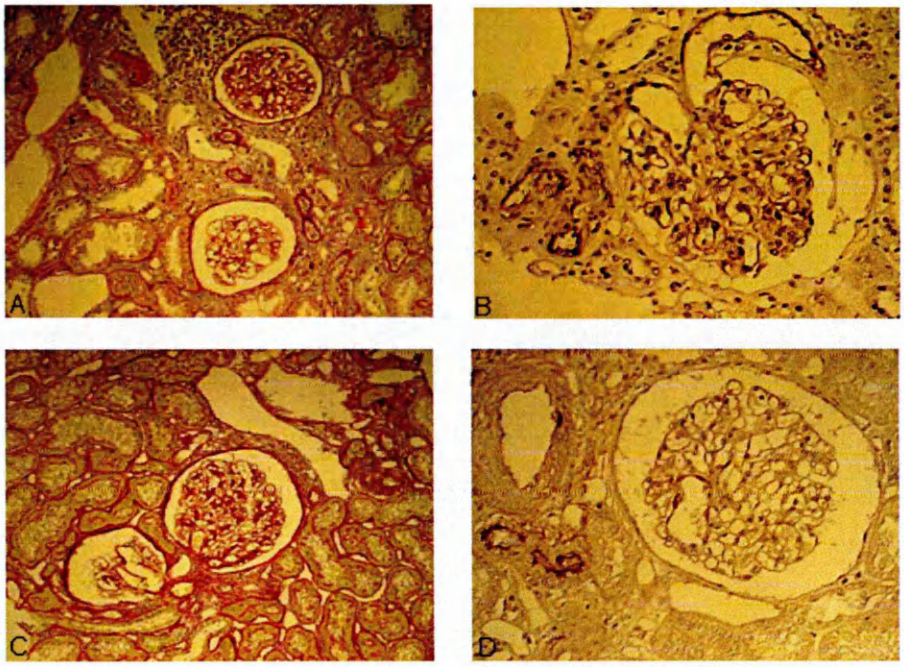
Graft function in the two treatment groups
Serum creatinine levels at different time points after transplant in the SRL (pink bars, n=11, n=10 at 24 months) and CsA (blue bars, n=10) group. Data are mean ± SE.

Figure 13



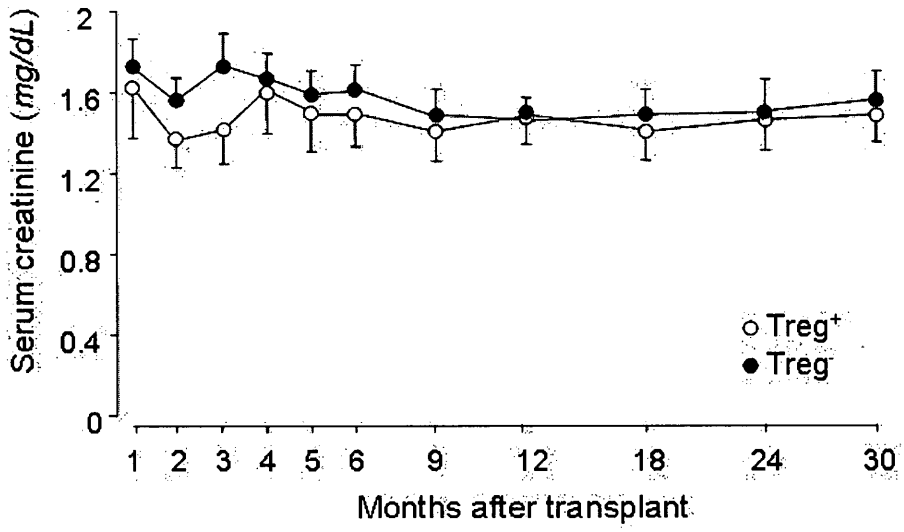
Time course of 24h proteinuria in the two treatment groups after Campath-1H induction
Median levels of 24h proteinuria in the two treatment groups in the follow-up period. Data from recipients of SRL plus MMF maintenance therapy are pink circles (n=11, n=10 at 24 months), data from recipients of CsA plus MMF are blue circles (n=10).

Figure 14



Graft histology
Representative photomicrographs of renal histology (A and C) and C4d staining (B and D) in biopsies taken 2 years post-transplant from patients receiving SRL (A and B) or CsA (C and D). Original magnification, x200 (A-C), and x400 (B-D).

Figure 15



Graft function according to Treg counts.
Serum creatinine profiles in patients with Treg counts higher (open circles, n=10) or lower (closed circles, n=11) than the median value at 12 months after transplant.

Figure 16

Discussion

In the second part of the study, we found that increased number of circulating Treg after Campath-1H induction and SRL maintenance therapy did not confer appreciable protection against development and progression of chronic allograft injury in renal transplant recipients. Actually, patients on SRL - who had higher circulating Treg compared to those on CsA - tended to have more severe histology changes consistent with chronic allograft rejection, faster GFR decline and some excess of persistent proteinuria.

Serum creatinine levels were relatively stable in both treatment groups during the whole follow-up period, although patients on SRL therapy had a slight trend toward a progressive increase. The lack of any significant difference in serum creatinine levels between the two treatment groups at any time-point after transplant might be at least partially explained by the small number of patients included in the study and to the relatively poor specificity of serum creatinine as a parameter for graft function [216]. Thus, to better evaluate graft function and its changes over time, we performed repeated (every 6 months after transplant) direct measurements of GFR and RPF by iohexol and PAH clearance, respectively. These are gold standard techniques that allow a precise and accurate evaluation of renal function [217]. Slopes of GFR changes over time showed that SRL patients had a significantly faster renal function decline as compared with CsA patients. Of note, yearly reduction of GFR in the CsA group was even lower than the physiological decline in healthy subjects after 40 years of age, *i.e.* 1 ml/min/1.73 m^2 [218]. A similar picture was found evaluating the RPF slopes, whose decline was remarkably although not significantly faster in the SRL than in the CsA group. In line with the above results, proteinuria higher than 0.5 mg/24h was slightly

more frequent among SRL than CsA patients. Importantly, these findings might translate into significantly different outcomes in the longer term. Indeed, both an high rate of GRF decline [219] and the onset of proteinuria [220] represent strong predictors of poor graft survival and function in the longer term.

The trend to worse renal function in the SRL group was associated with more severe histology changes at the per-protocol biopsies performed at 2 years after transplant. In particular, grafts from SRL patients showed more important tubulo-interstitial fibrosis, interstitial inflammation and arteriosclerosis than CsA treated ones. Also glomerular abnormalities were more severe in the SRL group. This translated into numerically higher CADI score for SRL as compared with CsA patients. Importantly, tubular staining for C4d was significantly more intense for SRL patients. C4d deposition in the peritubular capillaries of the graft has been correlated with the lesions of transplant glomerulopathy and with circulating alloantibodies [221]. As in our patients, the functional counterpart of these histology findings is often represented by the onset of proteinuria (generally of a low grade) and progressive renal dysfunction. In a retrospective study on 80 patients with histological diagnosis of chronic allograft nephropathy, those who had Cd4 positive staining in peritubular capillaries developed more frequently anti-HLA antibodies and had a significantly lower death-censored graft survival at 4 years as compared with those with negative staining (50% vs. 87%, P=0.002) [221]. Whether $Cd4^+$ staining was associated with anti-HLA antibodies and their potential impact on graft outcomes in our series of Campath-1H patients has not been evaluated. Notwithstanding, the present functional and histology data suggest that maintenance therapy with low-dose SRL and MMF may provide a less effective graft

protection than low-dose CsA plus MMF in renal transplant patients receiving induction with Campath-1H.

Finding that graft function and structure were better preserved in CsA treated patients was however unexpected, especially considering the renal toxicity of calcineurin inhibitors. Indeed, renal biopsy studies among nonrenal organ recipients with chronic kidney disease have shown that calcineurin inhibitor-related injury is a common finding [222]. Histopathologic findings include interstitial fibrosis with a "striped" appearance, nodular arteriolar hyalinosis, and, later, tubular atrophy with glomerulosclerosis and arteriosclerosis [37]. Hence, calcineurin inhibitor avoidance has been claimed as a strategy to improve the outcomes of the graft [223]. In our series, however, per-protocol biopsies from CsA treated-patients showed no sign of calcineurin inhibitor toxicity, possibly as a consequence of the small used doses of calcineurin inhibitor. Notably, CsA doses slightly declined over time and this might at least partially account for the improved RPF and GFR. Indeed, CsA exerts a vasoconstrictor effect, which may impair renal function. Thus, a progressive reduction of CsA over time might have improved glomerular hemodynamics and function.

Conversely, blood levels of SRL remained substantially stable over the whole follow-up period. Though initially considered devoid of any nephrotoxic effect, recent reports have been published showing that SRL might actually have renal toxicity. Indeed, a considerable number of *de novo* kidney transplant recipients on SRL-based immunosuppression [117, 224, 225] and of patients converted from calcineurin inhibitors or azathioprine to SRL due to cutaneous squamous cell carcinoma or chronic allograft dysfunction [226, 227], have been reported to develop proteinuria and glomerulopathy, both of which may predict lower allograft survival [220]. SRL toxicity

might therefore account for worse graft outcomes of patients in this treatment arm. This, however, seems extremely unlikely, since treatment was titrated to SRL blood levels (5 to 10 ng/ml) that are consistently lower compared to those associated with chronic nephrotoxicity (10 to 18 ng/ml) [224, 226]. Thus, other factors must be taken into consideration.

Finding that more severe structural and functional changes were associated with a significantly more intense C4d staining of the graft tissue, lend support to the possibility that the worse outcome of SRL-treated patients could be explained also by immune mechanisms resulting in more severe chronic allograft rejection. This would imply that in SRL-treated patients increased Treg cell count did not confer appreciable protection from the development of chronic alloimmune response. At variance, a specific Treg independent, protective effect of low-dose CsA against graft injury could explain the good long-term outcome of CsA-treated patients [228].

Finding that the increased percentage of Treg in SRL patients did not translate into an improved renal function was however unpredicted. Indeed, a large body of evidence is available that $CD4^{+}CD25^{high}$ Treg cells have potent immune regulatory effect *in vitro* [229, 230] and studies in experimental models of organ transplantation convincingly showed a protective effect of these cells against acute rejection *in vivo*. Anecdotal *ex vivo* findings in two kidney transplant patients have also suggested that Treg may play a role in preventing alloantigen epitope shifting, which is implicated in the ongoing immune activation contributing to chronic rejection [162]. Moreover, data from kidney transplant patients showed that the presence of Treg was associated with better outcomes, including a reduced risk of acute rejection [231] and better renal function in the long-term [167]. However, most of these results were obtained from patients on

different immunosuppressive regimens, at different time points after transplant, and not prospectically followed-up. In our series, the incidence of acute rejection was extremely low in both treatment groups, thus it does not allow to clearly state whether higher Treg levels might have played any role in the prevention of acute alloimmune response. However, progressive renal function worsening, combined with histology signs of humoral chronic rejection suggest that Treg did not provide any clear advantage in chronic graft protection.

The lack of any protective effect of Treg on the graft was confirmed by the absence of any significant correlation between the percentage of circulating Treg at 12 months and CADI histology score, GFR decline, and proteinuria levels at the last visit in the study group as a whole, as well as in each cohort considered independently. To further confirm this finding, we stratified patients according to the relative value of circulating Treg at one year after transplant above or under the median value. Again, outcomes of patients with higher circulating Treg levels were not better that the ones found in patients with lower levels.

Why SRL-treated patients were not protected from chronic allograft rejection despite the enhanced expression of Treg remains matter of speculation. One possibility is that the number of circulating Treg, although increased in the SRL group as compared to pre-transplant values, did not reach the threshold level to properly suppress the complex pathways of T effector cells, and thus to limit chronic graft injury. In murine models of spontaneous autoimmunity or genetically engineered mice, the Treg cells function normally but the T effector cells are resistant to Treg-mediated suppression when the phosphatidylinositol 3-kinase (PI3K)-Akt pathway is hyperactivated [232-234]. Costimulatory receptors and T cell receptor (TCR)-stimulation activate the PI3K-Akt

pathway in T lymphocytes which promote various cell responses associated with cell division including the inactivation of cell cycle inhibitors, and the induction of cyclin and cytokine gene expression [235]. These are early events in T cell activation that are not suppressed by SRL, which actually inhibits the downstream target protein kinase mammalian target of rapamycin (mTOR) [236]. A complementary explanation is that the local cytokine inflammatory milieu may inhibit the Treg immunomodulating activity in the graft. In support of this possibility is the evidence that in an experimental model of autoimmune encephalomyelitis, autoantigen-specific natural Treg accumulate in the central nervous system, but fail to effectively control the autoimmune reaction due to their exposure to locally released inflammatory cytokines, including IL-6 [237]. Notably, IL-6 renders naïve T cells resistant to suppression and enables the initiation of an immune response also in the presence of Treg [238]. In the context of the graft, alloreactive T cells themselves may be the source of IL-6 [239]. Moreover, in an inflammatory milieu, Treg may release TGF-β which, combined with IL-6, might promote the expansion of Th17 cells [240], a relatively new T cell subpopulation possibly involved in the rejection of the graft. This might explain why the potent *in vitro* activity of circulating Treg does not translate into an effective prevention the immune response inside the graft. Conversely, Treg activity might eventually result into an injurious effect through the induction of Th17 expansion. Evidence is also available that cells capable of rejecting the graft definitely remain after Campath-1H therapy [145]. In particular, post-depletion T cells are predominantly effector/memory T cells that expand in the first month after renal transplantation [173]. As shown in the previous chapter of results, also in our patients most residual T cells after Campath-1H actually expressed the $CD45RO^+$ memory-like marker. Like in the autoimmune experimental

models, reactivity of these T effector/memory cells might overwhelm the beneficial immune regulation of Treg [94]. Thus, in SRL-treated transplanted patients T effector cells, continuously activated by alloantigens and by intragraft inflammatory cytokines, could be less sensitive to regulatory T cell suppression.

A critical step in the pathogenesis of interstitial fibrosis in chronic allograft injury is epithelial-to-mesenchymal transition (EMT), whereby renal tubular epithelial cells change phenotypically and functionally into myofibroblasts [241]. The factor most capable of inducing and completing EMT is transforming growth factor-β (TGF-β) [242]. A possible candidate for TGF-β production during persistent inflammation (as it occurs in kidney transplantation) is the Treg cell which expresses the αE(CD103)β7-integrin allowing adhesion to epithelial cell E-cadherin [212, 243]. Importantly, the expression of CD103 is also associated with the presentation of membrane-bound TGF-β [244]. On this line is the recent evidence that $CD103^+$ T cells are bound to biliary duct epithelium in patients with primary biliary cirrhosis [245]. Thus, as alternative but not exclusive explanation of the present findings, Treg recruited into the graft might indeed activate tubular epithelial cells to trans-differentiate into myofibroblasts and ultimately promote interstitial fibrosis.

Beside efficacy parameters, in our study we also evaluated the safety profile of the two maintenance immunosuppressive regimens. Importantly, Campath-1H infusion was very well tolerated in all the patients. Adverse events were relatively mild and similarly distributed between the two groups, suggesting that different Treg counts did not affect either the risk of acute rejection or the susceptibility to opportunistic infections.

Blood pressure control and lipid profile were similar in the two cohorts, although SRL patients tended to have higher levels of tryglicerides and total cholesterol. Negative

effect of SRL on lipid metabolism is well known [246]. Indeed, SRL has been shown to increase apoB-100, apo C-II, apo C-III, and hepatic VLDL cholesterol production and to decrease heparin-induced LPL activity [247]. Thus, SRL appears to increase production of trygliceride-rich lipoproteins and prevents their breakdown.

Our present findings must be taken with caution since, because of the limited sample size and the reduced statistical power, the possibility of random effects cannot be definitely excluded. This may also explain why comparative analyses failed to detect significant differences in main functional and structural outcomes between the two treatment groups.

However, finding that all considered outcome parameters - histology score, GFR, proteinuria and C4d expression - consistently failed to show any trend to improved long-term outcome in SRL-treated patients with increased Treg count, provides a reasonable evidence of the robustness of our data.

Our data may have potentially important implications both for clinical management of kidney transplant patients receiving Campath-1H induction and for future research on Treg in the clinical transplant setting. This was the first study formally comparing two different low-dose maintenance immunosuppressive regimens after Campath-1H induction. Although the number of patients included in the study was relatively small, our data suggest that low-dose CsA combined with MMF conferred a similar protection against acute rejection than low-dose SRL, but was associated with a trend toward better graft function and lower histology injury at the 2 year per-protocol biopsy.

Importantly, the use of low doses of CsA was not associated with any sign of calcineurin inhibitor toxicity. Conversely, immunosuppressive therapy with SRL was associated with a trend toward faster renal function decline and worse graft hystology.

Regardless of the mechanisms involved – SRL nephrotoxicity and/or uncontrolled immune response –, our present results suggest that maintenance therapy with low-dose CsA and MMF might provide better outcomes than low-dose SRL and MMF in renal transplant patients receiving Campath-1H induction. Importantly, the presence of increased proportion of Treg among circulating lymphocytes in SRL patients did not provide any significant benefit on graft outcome, indicating that the role of these cells in the clinical transplant setting might be less evident than that suggested by experimental evidences in animal models.

FINAL DISCUSSION AND CONCLUSIONS

In the present study, we showed that a single 30 mg infusion of Campath-1H was able to induce a profound and long-lasting depletion of peripheral lymphocytes in renal transplant recipients on maintenance immunosuppression with low-dose of SRL or CsA, both combined with low-dose MMF. The two low-dose immunosuppressive regimens provided an effective protection against acute rejection and a good safety profile.

Importantly, SRL and CsA were associated with different phenotypic and functional patterns of circulating lymphocytes. In particular, SRL promoted the expansion of functionally active $CD4^+CD25^+FOXP3^+$ Treg, whereas CsA therapy was associated with increased numbers of $CD8^+CD28^-FOXP3^-$ T cells, whose functional characteristics are however still unclear. *In vitro* functional evaluation of peripheral lymphocytes, harvested at 1 and 2 years from transplant, showed decreased reactivity against donor cells in both treatment groups. On the other hand, anti third party response was suppressed in SRL and almost intact in CsA patients as compared with pre-transplant levels.

In the long-term, higher percentage of circulating Treg among SRL patients did not translate into improved clinical outcomes. Conversely, patients on CsA tended to have better graft function and lower histology injury in the per protocol graft biopsies performed at 2 years after transplant.

At the best of our knowledge, this was the first randomized, controlled, prospective trial comparing the immune characteristics and clinical outcomes of two different low-dose maintenance regimens of kidney transplant patients undergoing lymphocyte depletion through Campath-1H induction.

Lymphocyte depletion has long been recognized as a mean of preventing allograft rejection and has been pursued as a therapeutic strategy since the earliest days of transplantation [197]. Initially used to control immune response in patients at increased risk of acute rejection, it was subsequently proposed as a tool to minimize maintenance immunosuppression in patients at standard risk [122, 248]. Indeed, experience in non-human primate models of transplantation suggested that it might promote tolerance toward alloantigens [106].

With this background in mind, Campath-1H has been used in different organ transplants [126]. So far, however, clinical experience with Campath-1H has been largely limited to uncontrolled pilot trials and single-center experiences [249]. At the time our study was designed, the presumed dominant mechanistic effect was a reduction in T-cell precursor frequency limiting T-cell activation during the period of recovery from ischemic injury [136]. More recently, evidence came out showing that the pro-tolerogenic effect of Campath-1H may at least partially depend on the promotion of Treg expansion [175]. However, different maintenance immunosuppressive regimens may differently affect lymphocyte number and function, thus inducing or inhibiting Treg expansion and/or immune activity [114]. This might account for different outcomes reported with different maintenance immunosuppressive regimens after Campath-1H.

Thus, comparing different outcomes and immune fingerprints associated with different maintenance immunosuppressive regimens after Campath-1H induction might be of major importance to define the best maintenance immunosuppressive strategy and understand immune mechanisms potentially useful to design new pro-tolerogenic clinical protocols.

In the present study, we randomized recipients of a first single kidney transplant undergoing Campath-1H induction to maintenance therapy with low-dose SRL or CsA, both combined with low-dose of MMF.

Our working hypothesis was that SRL therapy would promote the expansion of $CD4^+CD25^+FOXP3^+$ T cells [177] and, considering their potential effect in controlling the immune response [250], we supposed that they might improve graft outcomes by preventing alloimmune injury. The present results, however, challenged our initial assumption. Indeed, although SRL therapy was associated with Treg expansion, this did not translate into any clinical advantage. Though because of the small number of patients included, the study might have had insufficent power to detect all but a large effect, our results do not support the idea that higher numbers of circulating Treg improve graft outcomes. Indeed, in spite of increased Tregs, SRL patients had a trend to even worse renal function and structure at 2 year per-protocol biopsy.

Our present results suggest that in the clinical setting the role of Treg might in fact be less important than in animal models of transplantation. Indeed a large body of evidence consistently showed that Tregs can induce and maintain immune tolerance and have the capacity to facilitate antigen-specific long-term graft survival successfully in animals receiving allogeneic organ transplants [229, 251]. Thus, the development of approaches to generate alloantigen reactive Tregs has been thought as an effective tool to induce tolerance in the clinical setting. Early clinical observations of Treg in human transplantation include reports of positive correlation between graft survival and circulating Treg in lung [252], liver [253] and kidney [254] allograft patients. $CD4^+$ Treg have also been reported in patients who developed spontaneous tolerance to liver [255] or kidney [256] allografts and in peripheral blood of human liver transplant

recipients weaned from immunosuppression [257]. A clear relationship between the levels of circulating Treg and graft outcomes is however not a uniform finding. This might be at least partially explained by the fact that circulating cells may not reflect intragraft events. Moreover, the redundancy and complexity of mechanisms of rejection underlies the difficulty in identifying biomarkers for prediction of graft outcome, and the induction of tolerance [258]. Also, potential local control mechanisms within the graft include not only several population of lymphocytes [88], but also mast cells [259] and monocytes [135]. Given this complexity, it is not surprising that no definite correlation between circulating Treg number and graft outcomes could be identified in our series. Indeed, at 2 years follow-up, non-alloimmune and/or non-inflammatory processes may well supervene to influence outcome, making associations even more difficult to detect [260]. In addition, a variety of individual factors, including sex, age and a range of gene polymorphisms potentially affect graft function and structure [260]. Notwithstanding, our results raise a concern about pro-tolerogenic strategies aimed at inducing donor-specific hyporesponsiveness through the promotion of Treg. Indeed, finding no positive trend between the number of circulating Treg and improved graft function or structure suggests that the role of these cells might not be so crucial in graft immune protection as previously believed, at least in human kidney transplantation.

Notably, we found that Campath-1H treated patients on low-dose CsA as maintenance immunosuppression showed donor-specific hyporesponsiveness and that anergy was the main mechanism at the basis of their low reactivity against donor antigens. Although recent years have seen a surge of interest around Treg, anergy might in fact represent an alternative important mechanism to promote tolerance [261, 262]. Indeed, in renal transplant patients with stable graft function, anergy has already been reported as a way

to promote hyporesponsiveness toward the donor [262]. It should be however taken into consideration that lymphocyte alloreactivity of all our patients was tested on ongoing maintenance immunosuppression. As anergy can be easily reverted by IL-2 addition, hyporesponsiveness toward donor cells in CsA patients might rely on maintenance therapy with an immunosuppressant that blocks IL-2 pathways [263]. Thus, CsA dose reduction or occurrence of inflammatory conditions such as infections, may result into an increased IL-2 production and thus revert the anergic state of these cells, eliciting an immune response against the graft.

Our findings challenge the hypothesis that Campath-1H is an intrinsic generator of Treg, as only SRL maintenance therapy was associated with the expansion of these cells. However, an intriguing finding of the present study was that maintenance immunosuppression with CsA was associated with the expansion of $CD8^+CD28^-$ T cells. Their negative expression of FOXP3 let us exclude they represented the same cell population isolated by Cortesini et al., which act by inducing endothelial and dendritic cells to express ILT-3 and ILT-4 inhibitory signals, thus making them tolerogenic [89]. Interestingly, a recent work by Trzonkowski et al. showed that, in renal transplant patients treated with Campath-1H, repopulating $CD8^+$ T cells were mainly of immunosenescent $CD8^+CD28^-$ phenotype and were able to suppress proliferation of $CD4^+$ T cells [174]. The Authors hypothesized that expanded $CD8^+CD28^-$ T cells might compete for 'immune space' with $CD4^+$ T cells, suppressing their proliferation and therefore delaying $CD4^+$ T cells recovery. Although we did not test immune function of $CD8^+CD28^-$ cells from CsA-treated patients, our results suggest that these cells might have played a role also in our cohort of patients. Further studies are however needed to clarify this point.

Another major contribution of the present study was finding that, when used at lower than conventional doses, CsA displays a relatively safe profile. Indeed, at the dosage employed in our protocol, it might provide enough immunosuppression to efficiently control both cellular and humoral allogeneic response, while exposing the kidney to minimal toxicity. Notably, also other well known adverse effects of CsA, including hypertension and glucose and lipid metabolism impairment, were relatively modest. Conversely, low-dose of SRL may have not been enough to control immune response, in line with the positive C4d staining in peritubular capillaries from patients in this treatment arm, which can be taken as a marker of humoral alloreactivity. On the other hand, even low doses of chronic SRL therapy might have negatively affected graft function, possibly even more than chronic use of calcineurin inhibitors. A growing body of evidence has suggested that SRL might indeed exert a nephrotoxic effect. Studies are available showing that SRL therapy is associated with the occurrence of proteinuria and this might be due to a direct toxic effect on podocytes [264].

Although calcineurin avoidance has been extensively claimed as a tool to improve renal graft outcomes, the largest randomized prospective trial performed so far, the SYMPHONY study, actually showed that low-dose CsA maintenance therapy provided better graft outcomes than low-dose SRL in kidney transplantation [265]. In this trial, 1,645 renal transplant recipients were randomly assigned to receive standard-dose cyclosporine, mycophenolate mofetil, and corticosteroids, or daclizumab induction, mycophenolate mofetil, and corticosteroids in combination with low-dose cyclosporine, low-dose tacrolimus, or low-dose sirolimus. Patients on low-dose calcineurin inhibitors had the lowest rate of acute rejection, and a trend toward better graft survival and function[265]. This finding highlights that calcineurin inhibitors represent crucial

molecules for immune suppression in transplantation, at least when non-depleting agents are used as induction therapy.

The beneficial effect of calcineurin inhibitors might be even more relevant in regimens including lymphocyte depletion. Indeed, after initial removal, lymphocytes undergoing homeostatic proliferation express markers of memory T cells. These cells have a low activation threshold, thus they may increase the risk of acute rejection. Importantly, *in vitro* experiments showed that CsA exert an inhibitory effect on these cells, which has not been shown for SRL [173]. Thus, maintenance immunosuppressive therapy with CsA might control alloimmune response better than SRL, especially in transplant patients undergoing induction therapy with lymphocyte depleting agents. This is in line with a retrospective analysis of the Organ Procurement and Transplantation Network/United Network for Organ Sharing (OPTN/UNOS) database, showing that, among Campath-1H treated patients, maintenance immunosuppression with calcineurin inhibitor-based immunosuppression may improve graft and rejection-free survival compared to calcineurin inhibitor-free regimens [137].

In conclusion, our study showed that: 1. Campath-1H is a safe and effective tool to prevent acute rejection with minimal doses of maintenance immunosuppression, in kidney transplant recipients; 2. maintenance immunosuppression with low-dose SRL and MMF is associated with the emergence of $CD4^+CD25^+FOXP3^+$ Treg, but this is not paralleled by improved graft function or structure; 3. maintenance immunosuppression with low-dose CsA and MMF is associated with donor-specific hyporesponsiveness of peripheral leukocytes, and this is at least in part due to T cell anergy. This is associated with a trend to better graft function and structure.

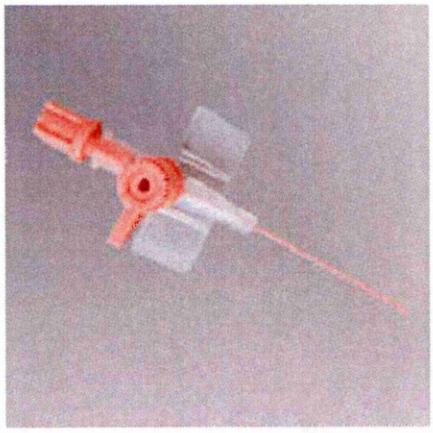
Our present findings suggest that lymphocyte depletion with Campath-1H might represent a useful strategy to minimize chronic immunosuppression in kidney transplantation, especially when CsA and MMF are used as maintenance drugs. The present results suggest that circulating Treg *per se* might be less relevant in transplant outcomes that previously thought. Further studies are however needed to clarify their impact in the clinical setting.

APPENDIX

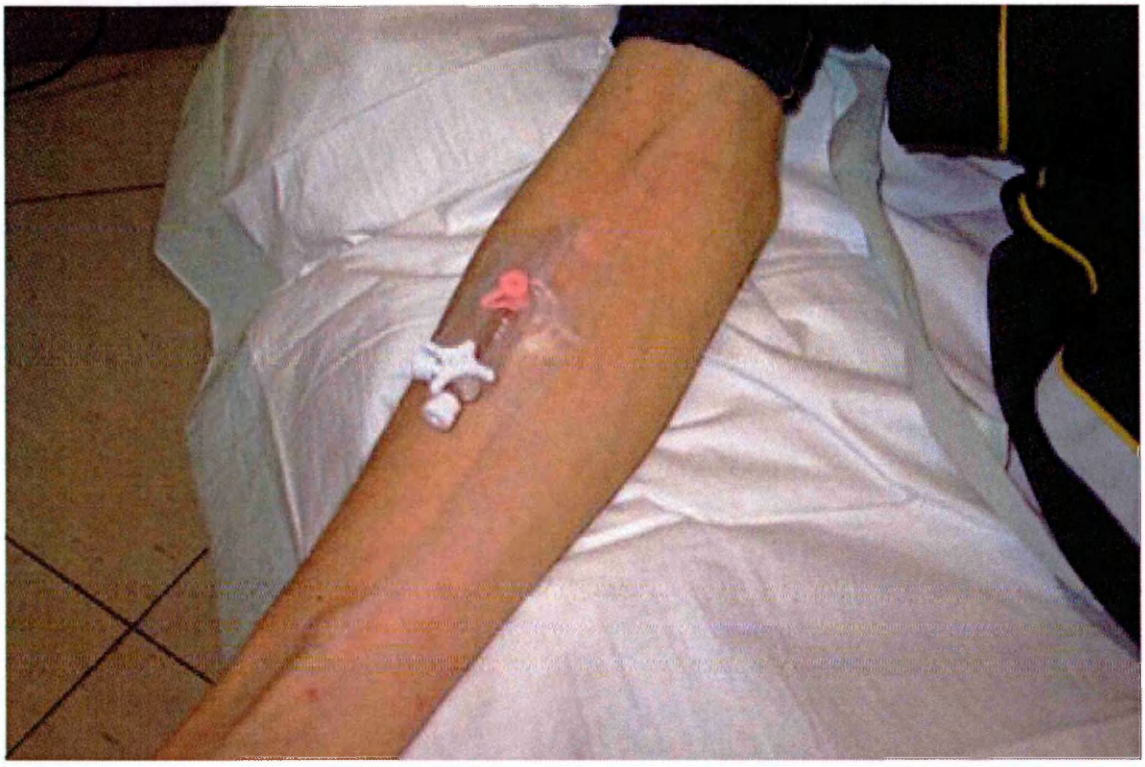
GFR and RPF measurement by plasma clearance of non-radioactive iohexol and paraaminohippuric acid (PAH)

Procedures

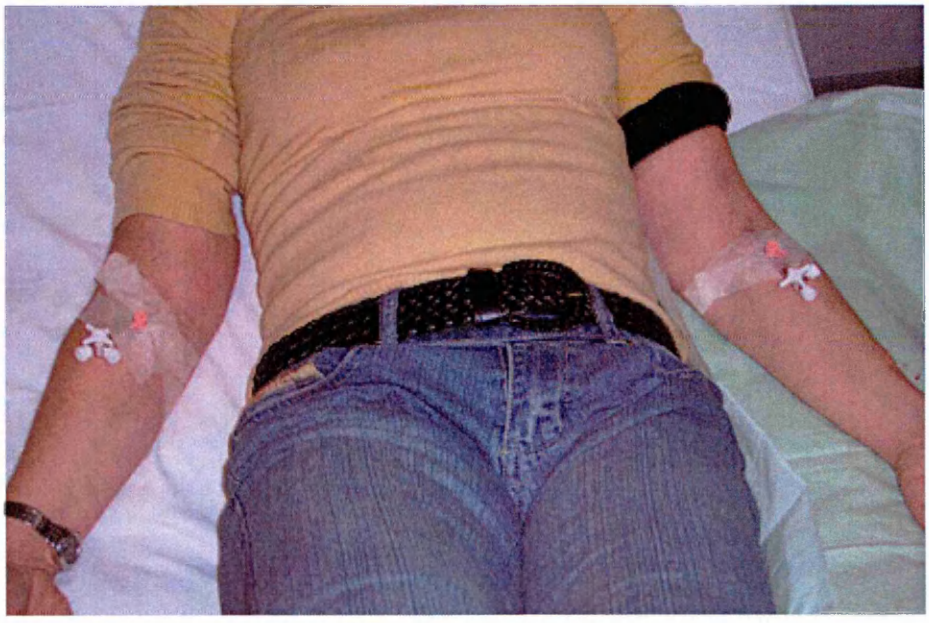
On the morning of the study, a catheter is positioned in an antecubital vein for the injection of the marker substances, and another in the contralateral arm for subsequent blood sampling.



Catheter



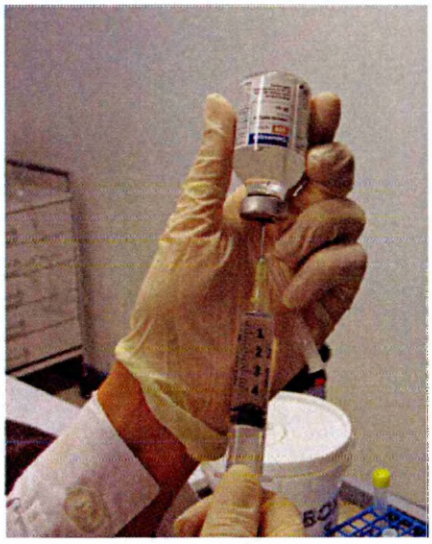
Catheter positioned in the antecubital vein



Catheters positioned in an antecubital vein for the injection of the marker substances, and in the contralateral arm for blood sampling.

After baseline blood sample collection for measurement of basal iohexol/PAH concentration (blank), 5 mL of Omnipaque containing 3.235 gr iohexol is slowly injected (2 minutes) into the injection catheter.

Drawing exactly 5 ml of iohexol



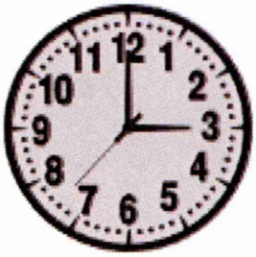
Syringe ready for 5 ml iohexol injection

Omnipaque solution (20 ml) can be used for more than one patient in the same day but not in different days.

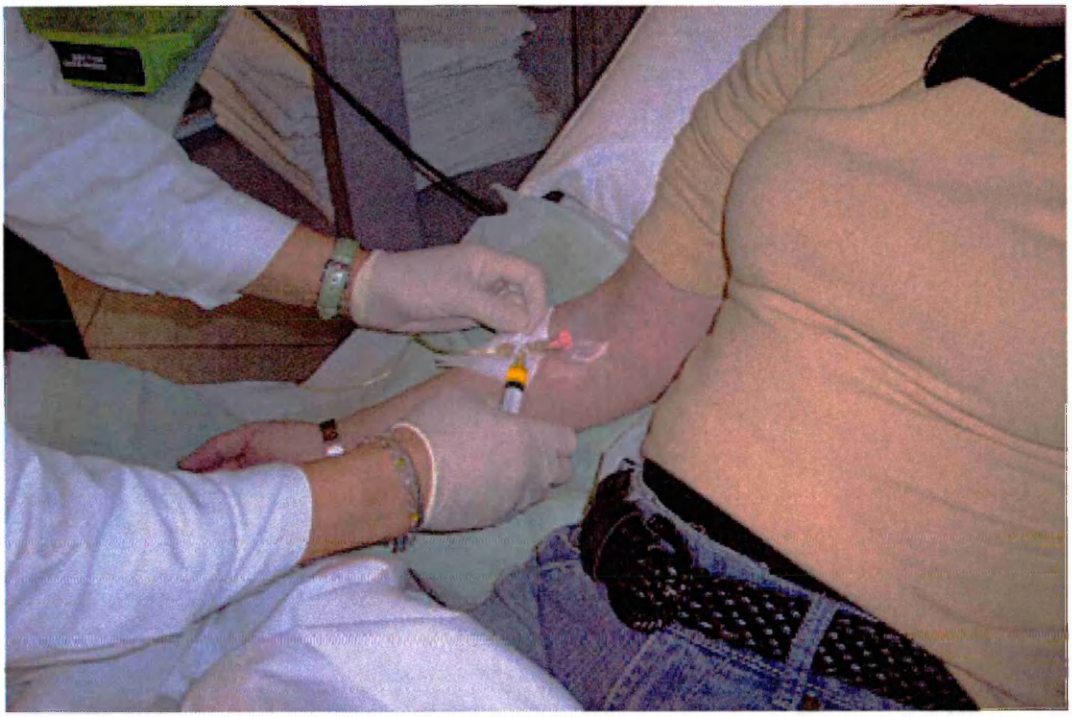
The catheter is then washed with 10 mL normal saline solution.

Washing the catheter with 10 ml saline

Note the exact time of injection (time 0) and use the same clock for timing throughout the procedure.



110 minutes later, a priming load of PAH solution at the dose of 8 mg/kg, adjusted for body weight in 5 kg increments, is injected in 1-2 minutes into the injection catheter, immediately followed by a timed constant infusion of 12 mg/min PAH with no adjustment for body weight, through a syringe-pump calibrated for precision of 0.04 mL/h. Again note the exact time of PAH injection. After the 180 min blood sampling the constant infusion of PAH is stopped and the catheter removed.



Injection of the priming load of PAH solution (dose = 8 mg/kg, in 1-2 minutes) adjusted for body weight in 5 kg increments

Constant infusion of 12 mg/min PAH solution (with no adjustment for body weight) through a syringe-pump

For Iohexol and PAH determination, blood samples are collected at different timepoints according to the expected GFR (as creatinine clearance):

a) for GFR > 40 mL/min sampling at:

120, 150, 160, 170, 180, 210, 240 minutes;

b) for GFR ≤ 40 mL/min sampling at:

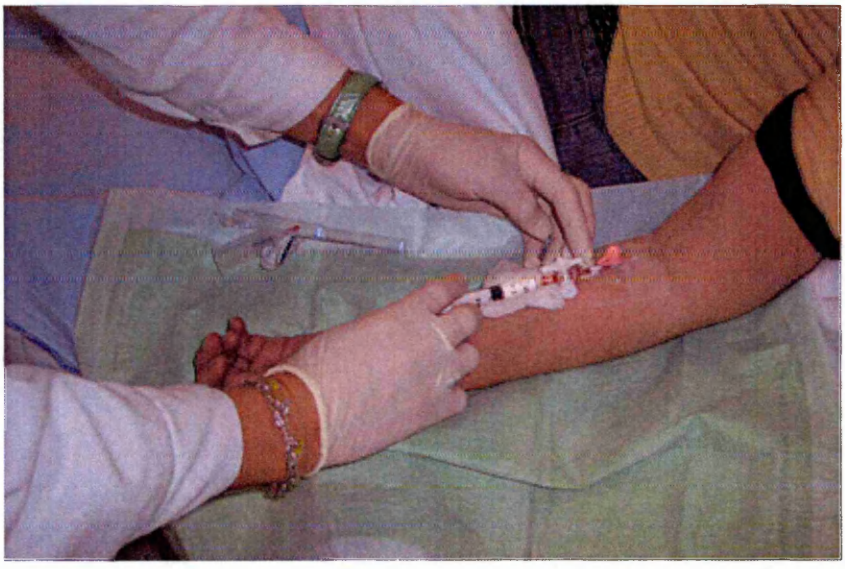
120, 150, 160, 170, 180, 240, 300, 360, 420, and 480 minutes.

after iohexol injection.

Before any blood sampling, discharge a blood volume at least equivalent to the catheter volume (1.5 mL) in order to avoid blood dilution with saline.

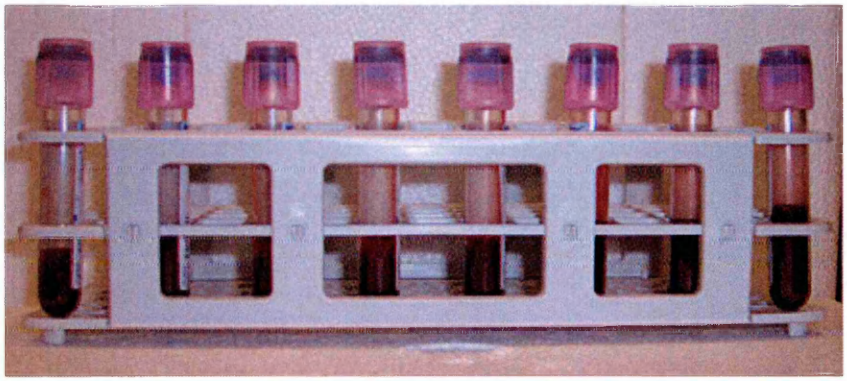
Discharging a blood volume at least equivalent Blood sampling to the catheter volume before sampling

Similarly, at the end of blood collection fill the entire catheter with saline solution.

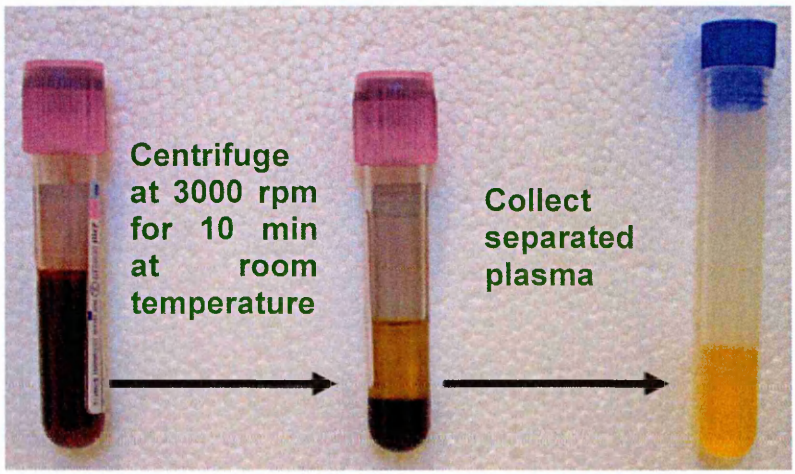


Filling the catheter with saline solution at the end of each blood collection

Blood samples can remain at room temperature until the end of the procedure before plasma separation.

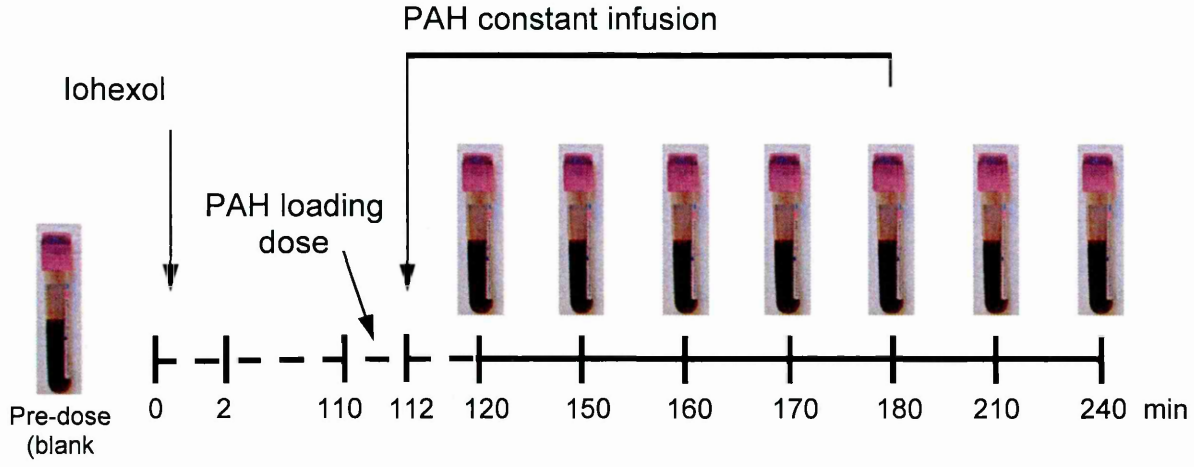


Blood samples (3 mL) are centrifuged at room temperature (3000 rpm for 10 minutes), the plasma collected and stored at -20 °C until assay.

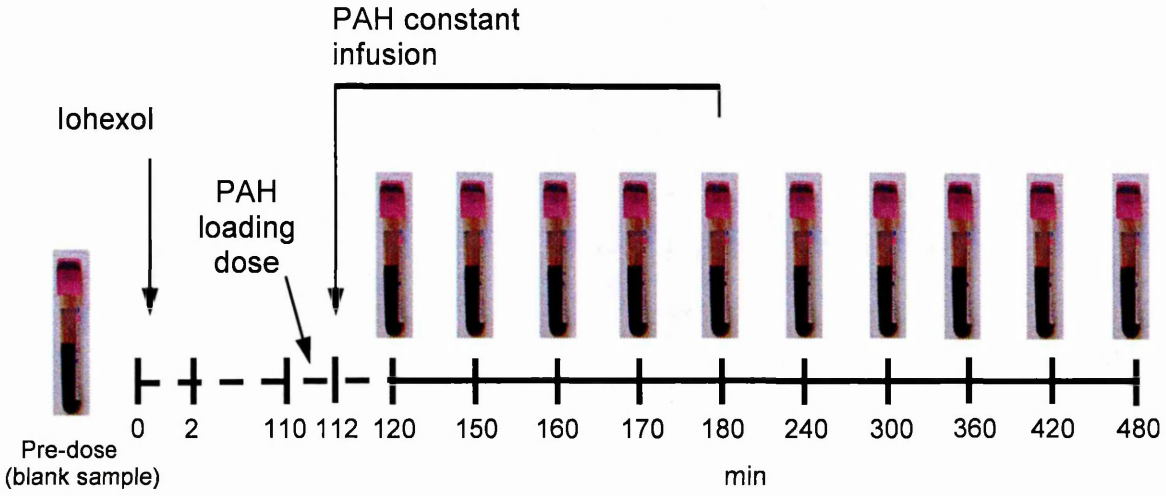


Flow charts for GFR and RPF measurement

- for an expected GFR > 40 mL/min:



for an expected GFR ≤ 40 mL/min:


PAH constant
infusion
Iohexol
PAH
loading
dose
Pre-dose
(blank sample)
0
2
110
112
120
150
160
170
180
240
300
360
420
480
min

Critical points of the procedures

1. To inject the exact dose of iohexol and PAH.
2. To obtain a constant infusion of 12 mg/min PAH.
3. To ensure that all fluid is injected intravenously and that there is no subcutaneous leakage.
4. To note the exact times at which samples are taken.
5. To draw blood not diluted with saline.

Preparing samples for analysis of iohexol and PAH

1. Centrifuge each samples at 3000 rpm for 10 minutes.
2. Pipette plasma from each sample into a tube.
3. Label, freeze, and store at -20° C until shipment.

Note:

a) the model assumes exactly 3.235 g iohexol being injected. Should the injected dose be different from this, please report the dose actually injected in order to correct the formula used for calculating GFR.

b) the model assumes exactly 8 mg/kg PAH, (adjusted for body weight in 5 kg increments), being injected and a timed constant infusion of 12 mg/min PAH being infused. Should the injected dose and/or the infusion rate be different, please report

the dose actually injected and/or the actual infusion speed in order to correct the formula used for calculating RPF.

c) the procedure implies all the markers are correctly injected. if subcutaneous leakage occurs, the procedure must be stopped and repeated a week apart.

Methods to determine iohexol and PAH concentration in the plasma samples and calculation of GFR and RPF values

Iohexol plasma concentration is determined by high performance liquid chromatographic (HPLC) method. Iohexol clearance is first calculated according to a one-compartment model (CL_1) by the formula:

$$CL_1 = \text{injected dose of iohexol/AUC}$$

where AUC is the area under the curve of the plasma concentration of iohexol. Then the value obtained is corrected according to Bröchner-Mortensen, in order to estimate the corrected GFR (plasma clearance) by using the formula:

$$GFR=(0.990778x\ CL_1)-(0.001218x\ CL_1^{\ 2})$$

GFR values is then normalized for body surface area and expressed as ml/min/1.73 sqm.

PAH concentration is measured by HPLC by using the same method for iohexol measurement. PAH clearance is calculated according to the formula:

$$RPF=Ro/CssPAH$$

where Ro is the infusion rate; CssPAH is the PAH plasma concentration at the steady state (i.e. mean of PAH plasma concentrations measured at 150, 160, 170, 180 minutes from iohexol injection)

Renal plasma flow, estimated by plasma clearance of PAH, is then normalized by body surface area, and expressed as ml/min/1.73 sqm.

LIST OF ABBREVIATIONS

6-MP: 6-mercaptopurine

AUC: area under curve

BM: bone marrow

CADI: chronic allograft damage index

CAN: chronic allograft nephropathy

CMV: cytomegalovirus

CsA: cyclosporine

DIC: diffuse intravascular coagulation

DTH: delayed-type hypersensitivity

ELISPOT: enzyme-linked immunospot

GBM: glomerular basement membrane

GFR: glomerular filtration rate

GVDH: graft versus host disease

HLA: humal leukocyte antigen

HPLC: high-performance liquid chromatography

LDA: limiting dilution assay

MAP: mitogen activated protein

MHC: major histocompatibility complex

MLR: mixed lymphocyte reaction

MMF: mycophenolate mofetil

mTOR: mammalian target of rapamycine

NFAT: nuclear factor of activated cells

PAH:p-aminohippurate

RPF: renal plasma flow

SRL: sirolimus

TLI: total lymphoid irradiation

Treg: regulatory T cell

UNOS: United Network for Organ Sharing

MATERIAL PUBLISHED OR ORALLY PRESENTED CONTAINING WORK DESCRIBED IN THE THESIS

Material published

1. Noris M, Casiraghi F, Todeschini M, Cravedi P, Cugini D, Monteferrante G, Aiello S, Cassis L, Gotti E, Gaspari F, Cattaneo D, Perico N, Remuzzi G. Regulatory T cells and T cell depletion: role of immunosuppressive drugs. J Am Soc Nephrol. 2007;18(3):1007-18.

2. Ruggenenti P, Perico N, Gotti E, Cravedi P, D'Agati V, Gagliardini E, Abbate M, Gaspari F, Cattaneo D, Noris M, Casiraghi F, Todeschini M, Cugini D, Conti S, Remuzzi G. Sirolimus versus cyclosporine therapy increases circulating regulatory T cells, but does not protect renal transplant patients given alemtuzumab induction from chronic allograft injury. Transplantation. 2007;84(8):956-64.

Material orally presented

1. Cravedi P, Casiraghi F, Todeschini M, Monteferrante G, Cugini D, Bontempelli M, Perico N, Noris M, Remuzzi G. Effects of Sirolimus (SRL) versus Cyclosporine (CsA) as maintenance therapy after Campath induction on T cell alloreactivity in kidney transplant recipients. *Am J Transplant* 6 (Suppl2): 171. 2006 Oral presentation at the World Transplant Congress, 2006, Boston.

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